DOCTORAL THESIS

“Long-term effects of prenatal and early postnatal environment on brain remodelling: focus on hippocampal volume and astroglia”

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"Long-term effects of prenatal and early postnatal environment on brain remodelling: focus on hippocampal volume and astroglia"

By

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Abbreviations

5HT-1A	5 Hydroxytryptamine -1A
ABC	Avidin Biotin Complex
ACTH	Adreno-corticotropic hormone
ANOVA	Analysis of Variance
BDNF	Brain derived neurotrophic factors
BLA	Basolateral amygdala
Brdu	5 –bromo 2 – deoxuxyuridine
BSA	Bovine serum albumin
CA1-CA3	"Cornu Ammonis" Latin for Ammon's Horn - Regions 1 to 3
CeM	Central amygdala
Cg	Cingulate cortex
CNS	Central nervous system
CRH	Corticotropin releasing hormone
DAB	3-4' Diaminobenzidine
Dex	Dexamethasone
DG	Dentate gyrus
DRN	Dorsal raphe nulcei
ED	Early Deprivation
EH	Early Handled

ELS	Early life stress
fmol	Femto moles
GABA	Gamma - aminobutyric acid
GC	Glucocorticoid
GD	Germinal day
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid receptor
HPA	Hypothalamo-pituitary-adrenal axis
HX	Hematoxylin
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
mRNA	messenger Ribonucleic acid
Nacc	Nucleus accumbens
NH	Non Handled
NPY	Neuropepdide Y
NSC	Neural stem cells
OCT	Optimum cutting tissue
OT	Oxytocin
PBS	Phosphate buffered saline
PND	Post natal day
pQCT	Peripheral quantitative computed tomography

PrL	Prelimbic cortex
PVN	Paraventricular nuclei
SAM	Sympathetic-adrenomedullary-system
SHRP	Stress hypo responsive period
SNRP	Stress non-responsive period
STr	Subiculum transition area
VMH	Ventro medial hypothalamus
WT	Wild type

Abstract of the thesis

The main aim of this thesis was to assess if early deprivation (ED) and glucocorticoid (GC) treatment exert long-term effects on the volume of the brain regions implicated in responses to stress, and if it associates with alterations in the distribution and structure of astroglia, which are known to support brain plasticity. This study also investigated the effects of prenatal dexamethasone (Dex) treatment on selected brain receptors, namely the oxytocin and 5-HT1A receptors, as they are implicated in the regulation of responses to stress. In addition, *in vitro* effects of Dex on neural stem cells were studied, in order to explore the drug effects on cell proliferation and differentiation, and on glial cell markers.

Unbiased stereological estimation was employed to determine the regional brain volume, astroglial morphology and total cell count. Peripheral quantitative computed tomography (pQCT) technique was used to quantify total brain volume. Autoradiography technique was employed to visualise and analyse oxytocin and 5HT-1A serotonin receptor binding using selective radioactive ligands.

The results of the present study demonstrate that both ED and prenatal Dex exposure leads to long-term effects on hippocampal remodelling with volume losses and impoverished astroglial morphology in the form of reduced primary process length. The observed deterioration in astroglial morphology adds further evidence that astrocytic changes contribute to hippocampal volume losses, a phenomenon that deserves more research in the context of effects of corticosteroid overload and stress-related pathologies.

The present results also demonstrate that prenatal Dex induces long-term effects at the level of central neuroregulatory processes. Thus significant region- and sex-dependent reductions or increases in the oxytocin and 5-HT1A receptor binding were observed. The *in vitro* study has shown that Dex affects both proliferation and differentiation of GFAP positive NSCs with no toxic effects as such.

Overall, both early postnatal or prenatal manipulations that increase levels of stress and/or glucocorticoids as the chemical mediators of stress, lead to a long-term maladaptive brain remodelling with losses in the hippocampal volume, impoverishment of hippocampal astroglial morphology and changes in the properties of central regulatory receptors in the brain areas involved in the reaction to stress.

List of Publications

Publications – Papers accepted & in preparations

Shende V. H., Pryce C. R., Rüedi-Bettschen D., Rae T. C. & Opacka-Juffry J. (2012). Evidence for hippocampal remodeling as a long term effect of early life stress in the rat model of early deprivation. Recent Devel. in Brain Res., 1(2012): 1-19

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Publications – Conference contributions

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Shende V. H., McArthur S., Gilles G. E. & Opacka-Juffry J. (2011). Astroglial plasticity is implicated in hippocampal remodelling in adult rats prenatally exposed to dexamethasone. Society for Neuroscience Abstract. Washington, USA

Shende V. H., Molina-Holgado F., Opacka-Juffry J. (2011). Dexamethasone affects astroglial differentiation in neural stem cells: Role of endogenous interleukin-1 beta. British Pharmacological Society Abstract. London, UK

Shende V. H., McArthur S., Gilles G. E. & Opacka-Juffry J. (2011) Perinatal exposure to dexamethasone leads to a reduction in the hippocampal volume in rats: A stereological study. British Neuroscience Association Abstract. Harrogate, UK

Opacka–Juffry J., Shende V. H., Leventopoulos M., Mannar M. J., Russig H., Rüedi-Bettschen D. and Pryce C. R. (2011) Long-term effects of early life stress on brain remodelling – experimental studies. British Neuroscience Association Abstract. Harrogate, UK

Shende V. H., Rae T. C. & Opacka-Juffry J. (2010) pQCT - Tool for brain volume measurement. Federation of European Neuroscience Societies Abstract., vol.5, 207.3 - Amsterdam

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Chapter 1

General Introduction

This chapter consists of the background information and provides an introduction to the core topics of the thesis.

1.1 Stress

1.1.1 The concept

The very concept of stress evolved in the early twentieth century. Although over the decades, stress has been a major subject of research, there is no precise or straightforward definition of stress.

Stress affects the lives of most adults in developed countries in many ways. It is a major factor in rising health care costs in the United Kingdom. The Health and Safety Executive (HSE) statistics report that about half a million people in the U.K. experience work-related stress at a level they believe is making them ill. Furthermore, the overall cost of medical care, time lost from work, and workplace accidents come to over £15 billion per year (HSE report, 2009-10).

It was in the year 1936 when Hans Selye coined the term "Stress" and described it as a "general adaptation syndrome". He wrote: "Stress is the body's nonspecific response to a demand placed on it" (Selye, 1936; page 32). In the general adaptation syndrome, he described three periodic stages of the stress syndrome occurring under the stressful event, the general alarm reaction, resistance and exhaustion (Selye, 1936). This new concept provided a basis for researchers to extend their knowledge and its implications. Stress is a term most commonly used to describe experiences that are challenging emotionally and physiologically. Activation of adaptive responses induced by a physical, chemical or psychological factor (known as 'stressor') and leading to a disruption of body homeostatic processes is stress according to Sapolsky (2003). These

adaptive responses constantly attempt to alter the external environment in order to reduce the challenge to homeostasis.

Homeostasis is a concept introduced by Walter Cannon (1926). It is the physiological process, which maintains stable and constant internal environments, despite of the variations occurring in the external environments. The components of the internal milieu like pH, oxygen tension or body temperature are the important factors for the survival of an individual and cannot adapt as per external stressor. The mediators such as hormones, neurotransmitters, immune system, heart rate, blood pressure participate in the process of adaptation by fluctuating their levels (McEwen, 2000).

The brain is the key organ, which registers any real or perceived stressful situation. It is the master regulator of the neuroendocrine, autonomic and immune systems, also controls the behavioural and physiological responses that are produced (Figure 1.1). Along with the metabolic, cardiovascular and immune systems, the brain is also a target of stress. Within the brain, the hypothalamus is essential for autonomic and neuroendocrine responses to stressors. The limbic system of the brain, involved in memory, anxiety and decision making, is the prime target of stress and stress hormones. Stress hormones play a major role in mediating both adaptive and maladaptive responses, by interacting with specific aspects of the physiology of each tissue. A characteristic behavioural feature of a stress response is the classical "fight-or-flight" situation. The normal stress hormone response is crucial to survive in such situations instead of inadequate or excessive functioning, which is deleterious for health and survival.

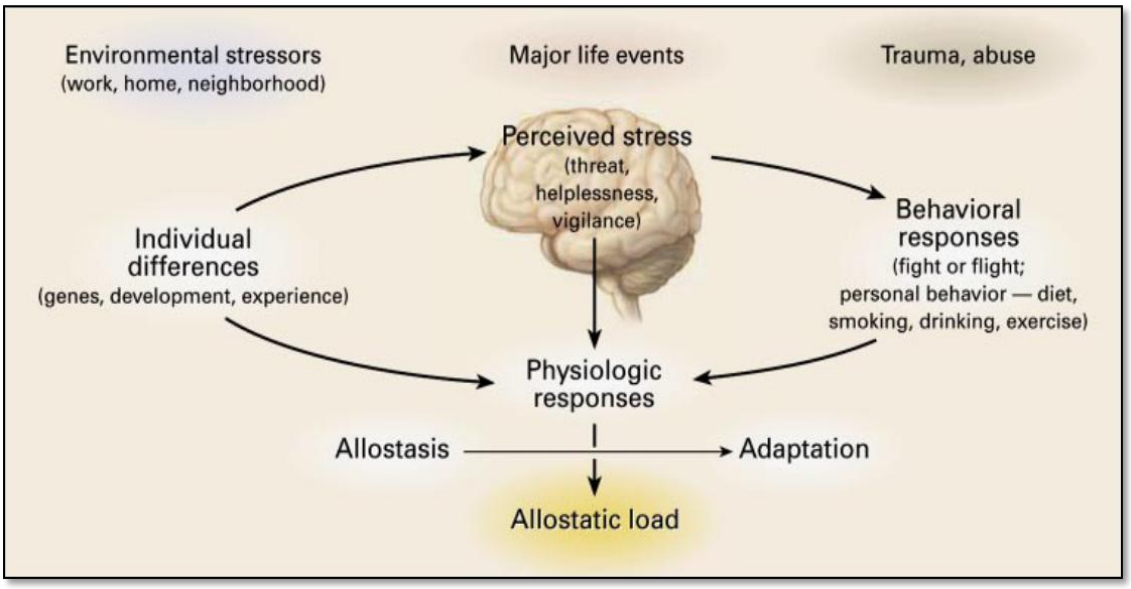

Figure 1.1 Central role of brain in allostasis, behavioural and physiological response during the event of stressful situation (Source: McEwen, 1998, page 3)

The perception of stress is influenced by an individual's experience, genetics and behaviour. In response to stress-inducing factors (stressors) the brain initiates the physiologic and behavioural responses, leading to allostasis and adaptation. When the stress exists for longer time, allostatic load can accumulate and the overexposure to mediators of neural, endocrine, and immune stress can have damaging effects on various organ systems, leading to disorders (McEwen, 1998; 2007).

Stress and an adaptive response to it have protective as well as detrimental effects, due to this nature introduction of the term 'allostasis' was defined by Sterling and Eyer (1988). Allostasis is "the process that keeps the organism alive and functioning, i.e. maintaining homeostasis or 'maintaining stability through change' and promoting

adaptation and coping, at least in the short run" (McEwen, 1998). The mediators, which are involved in the allostatic state are tissue mediators – corticotropin releasing hormone (CRH), pituitary hormones – adreno-corticotropic hormone (ACTH), glucocorticoids (GCs), cytokines, neurotransmitters – γ-aminobutyric acid (GABA) and neuropeptides – NPY (McEwen, 2002).

The consequence of the psychosocial or physiological adaptations is allostatic load. It is referred to as the price, which the body pays for being forced by adaptations caused by use of allostasis, particularly when the mediators are dys-regulated (not turned off when the stress is over or not turned on when they are needed) (McEwen, 1998 and 2007).

1.1.2 The stress response system: Hypothalamo–Pituitary–Adrenal axis and Sympathetic–Adrenomedullary system

As mentioned earlier, the stress response is the body's constant effort to right any physical or mental stressor to maintain physiological, mental and emotional harmony or homeostasis. The consequence of an individual not able to re-establish homeostasis is a disease. These homeostatic processes are maintained via the hypothalamo-pituitary-adrenal (HPA) axis, which is the primary responder to stress (De Kloet *et al*, 1999). Activation of the hormonal stress pathway (glucocorticosteroids) tends to be associated with depression, whereas the activation of the neural stress pathway (adrenaline) is more frequently correlated with anxiety (Reilly, 2006).

Stress responses in humans and animals lead to various biological and neuro-endocrine effects, such as excessive activation of the HPA axis with a release of glucocorticoids from the adrenal gland cortex and activation of the autonomic nervous system (ANS), but these depend on the level and duration of stress (Fuchs and Flugge, 1998). The extent of the stressor applied is determined by the parvocellular neurosecretory cells of paraventricular nuclei of the hypothalamus. Immediate release of the corticotropin releasing hormone (CRH) occurs into the portal vascular system, which stimulates the release of the adreno-corticotropic hormone (ACTH) via the anterior pituitary gland. ACTH then enters the blood circulation and stimulates the adrenal cortex to produce and release glucocorticoids (cortisol in humans and corticosterone in rats) (Kaufman et al, 2002; Swaab et al, 2005). At the same time, activation of the sympathetic-adrenomedullary-system (SAM) occurs as an immediate response to stress. SAM stimulates the adrenal medulla to release catecholamines (adrenaline and noradrenaline) initiating fight or flight response. GCs can pass through the blood brain barrier while adrenaline does not cross the blood brain barrier (Seckfort *et al*, 2008).

The HPA axis responsiveness is determined by the ability of the glucocorticoids to regulate CRH and ACTH release by binding to two corticosteroid receptors, glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). GRs are widely distributed in the brain and play a major role in stress responsiveness, as even at high levels of GCs there are still GRs sites available (De Kloet et al., 1991). MRs are majorly found in hippocampus and are less distributed in comparison to GRs. MRs have high affinity for GCs and play an important role during the ACTH inhibition (Reul and de Kloet, 1985; de Kloet et al., 1991; Young et al., 1998). Once the extent of stressor is

decreased, the negative feedback mechanism is activated at various levels of the system i.e. from the adrenal gland to the hypothalamus and other brain regions including the hippocampus and prefrontal cortex (Figure 1.2) (Lupien et al., 2009). This in turn lead to HPA axis shutdown and the system returns to homeostasis or normal stable point.

GRs in the hippocampus respond to GC release from the adrenal cortex and induce negative feedback inhibition of the HPA axis (McEwen, 2001b). Therefore, the hippocampus activates a GC-dependent negative feedback regulation of the HPA axis by inhibiting CRH and further ACTH release preventing excess glucocorticoid secretion (Jongen-Relo et al., 2002). Glucocorticoids play a major role in the HPA axis-mediated regulation in stress and stress related disorders within the brain mainly affecting the prefrontal cortex and limbic system, which includes the hippocampus and amygdala (Fuchs and Flugge., 1998).

The autonomic nervous system, cytokines, metabolic hormones and neuroendocrine systems that are involved in stress response are altered by the HPA axis activity, are also involved in the pathophysiological changes that occur in response to chronic stress, from early life experiences into adult life.

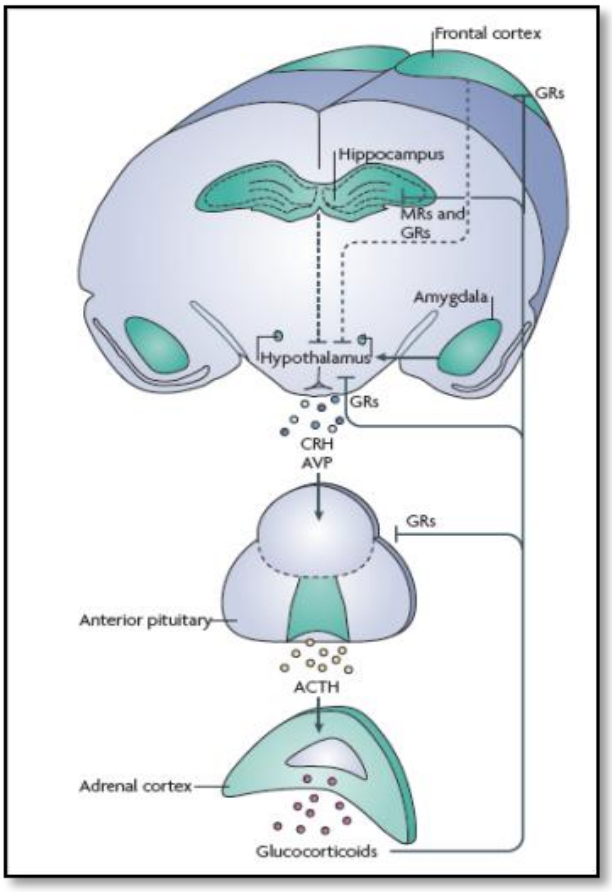

Figure 1.2 Hypothalamic Pituitary Adrenal axis system response of brain to the stress.

(Source: Lupien *et al.*, 2009)

(*MRs - mineralocorticoid receptors, GRs - glucocorticoid receptors, CRH – corticotropin releasing hormone, AVP – arginine vasopressin and ACTH – adreno-corticotropic hormone*)

The HPA axis pathway is activated under the stressful situation and is crucial in terms of the survival due to its homeostasis activity via a negative feedback mechanism. The above figure shows various key processes involved in the event of stress and body's reaction to it.

1.1.3 Acute and Chronic stress

Stress is often linked with negative or adverse life experiences, being perceived as bad or damaging. The extent of stressor determines the extent of the vulnerability. Stressors can be categorized as short term (acute) or long term (chronic). Experience of stress that is of limited duration is known as acute stress which can be 'good', and which leaves a sense of exhilaration and accomplishment in humans. Whereas experience which is prolonged or recurrent, irritating, emotionally draining and physically exhausting or dangerous, and beyond control is what Selye called 'bad stress' and which leads to deleterious effects. In other words, acute stress often leads to adaptive responses, which can maintain homeostasis. On the other hand, chronic stress leads to maladaptive responses, which are not able to maintain homeostasis leading to a pathopysiological cascade and may result in episodes of depression (De Kloet et al., 2005; McEwen, 1998 and 2007).

Acute stress is the reaction to an immediate threat, classically known as the *fight or flight* response. The threat can be any situation that is perceived, even subconsciously or falsely, as a danger. Common acute stressors include noise, isolation, hunger, infection or even remembering a dangerous event. Under most of these circumstances, once the acute threat has passed the relaxation response occurs where levels of stress hormones return to normal. Frequently, life poses ongoing stressful situations resulting in chronic stress. Common chronic stressors include ongoing highly pressured work, long-term relationship problems or financial insecurities. These results in the upregulation of the

stress hormones, which causes detrimental effects leading to a disease (Joëls, 2006; Joëls et al., 2006; McEwen, 1998).

Preclinical research has shown that acute stress can produce a beneficial effect on learning and memory, through the effects of glucocorticoids on the hippocampus and the cerebral prefrontal cortex, the key regions that control memory (McEwen, 1998; 2000). Acute stress also leads to an increase in the release of the neurotransmitter glutamate in the prefrontal cortex which supports working memory (Yuen et al., 2009). Chronic stress is linked with damaging effects on the normal brain functioning, which results in deficits of hippocampus-mediated learning and memory disruption of cell growth and neurogenesis (Joëls, 2006; McEwen, 2007; Joëls et al., 2006; Pardon and Marsden, 2008).

1.1.4 Life cycle model of stress

The brain is referred to as dynamic and unique due to its feature of adaptive plasticity. Compared to other organs, the brain is the slowest to develop. Prenatal stress exposure has programming effects on the development of brain regions such as the hippocampus, frontal cortex and amygdala. These brain parts belong to the so called limbic system, which is involved in the regulation of the hypothalamus-pituitary-adrenal axis (HPA) (Levine, 1994).

Early postnatal stress exposure can have damaging effects on the brain. Postnatal stress exposure in the form of early deprivation or maternal separation in childhood causes excessive release of glucocorticoids whereas exposure in the form of childhood abuse

leads to reduced secretion of glucocorticoids according to the model suggested by Lupien *et al*. (2009). Developing brain regions are susceptible to the effects of stress hormones, yet some areas undergo quick recovery during a particular time. The hippocampus is the key area related to memory formation and is under development stage for an initial two years of age in humans and is most prone to stress effects. While the amygdala continues to develop until late 20s. Hence, stress exposure in childhood and/or adolescence may cause changes in amygdala volume (Heim and Nemeroff, 1999; Lupien *et al*, 2009). Stress exposure during adolescence may also lead to changes in the frontal cortex volume when it is still under development (Teicher et al., 2006). Adolescence is the most critical period of development where the probability of stress exposure is higher due to protracted glucocorticoid response causing potentiation/incubation effects which may extend to adulthood. Most rapid decline in brain region commences during adulthood and aging due to proneness to stress hormones and which is why the brain is most susceptible to neurodegenerative diseases during aging (Barr et al., 2004; Lupien *et al*, 2009).

1.2 Early Life Stress

1.2.1 The concept and effects on the brain

The importance of the environment in the regulation of brain, physiology and behaviour has long been recognised in the biological, social and medical sciences, which leads to an important junction in the study of the causal relationships between physical and social environmental factors and neurobiological, physiological and behavioural phenotypes (Levine, 1960; Nemeroff, 1998; Pryce and Feldon, 2003). In humans, stressful early life experience occurs in the form of child abuse/neglect or parental loss which is a major risk factor of depression (Heim and Nemeroff 2001; McEwen, 2000). Studies have observed that low maternal care and avoidance of depressed mothers towards their children affect the normal neuronal development and HPA axis function resulting into neuropsychiatric or neurological disorders later in life (Francis and Meaney, 1999; Kaufman et al., 2000; Nicol-Harper et al., 2007).

In mammals, infancy is the period of life which is characterised by marked neuroplasticity and strong mother – infant interactions (Pryce *et al*, 2002). The normal postnatal development of the CNS of an infant depends on the mother – infant relationship (Pryce *et al*, 2005). The mother provides stimuli to rat pups not only through feeding but also through contact and warmth (Lehmann and Feldon, 2000). The maternal homeostatic regulation in infants is maintained via thermal - metabolic regulation which maintains oxygen consumption and heart rate at a normal stable level, nutritional regulation which is provided by milk and tactile regulation which maintains growth hormone and catecholamines (Pryce *et al*, 2005).

In the year 1962, Schapiro and colleagues conducted one of the earliest studies on postnatal effects of the HPA axis in rats during the first two weeks after birth and found very low blood levels of corticosterone and no overactivity of HPA axis when exposed to mild stressors. Hence, this period was named as stress non-responsive period (SNRP) (Schapiro et al., 1962). These studies were based on the measurement of the corticosterone levels in the blood. However, later studies by Schoenfeld and colleagues and Walker found HPA axis overactivity in rats exposed to ether fumes (Schoenfeld et al., 1980) suggesting HPA axis functioning depends on the time and type of stressor (Walker et al., 1990). Hence, the stress non-responsive period (SNRP) was renamed as stress hypo-responsive period (SHRP).

Rat pups are said to be in a stress hypo - responsive period (SHRP) during 2-14 postnatal days (PND) and show characteristic neuroplastic and neuroendocrine patterns (Pryce *et al*, 2002). The SHRP maintenance is dependent on the level of maternal care. Inadequate maternal care leads to the stimulation and dysregulation of the HPA axis activity because newborn pups have a high density of glucocorticoid receptors in the pituitary and activates the negative feedback inhibitory signals during early stages of life (Lehmann and Feldon, 2000). Animal models using disrupted maternal care are characterised with chronic physiological, neurobiological and behavioural effects in their adulthood (Pryce and Feldon, 2003; Pryce *et al*, 2005). Early life manipulation in rodents and primates can model the relationship between early life stress and depression. Manipulation in early life reflects human traits such as neglect like behaviour and reduced motility towards reward (Pryce *et al*, 2005; Rüedi-Bettschen *et al*, 2005). Marked changes in behaviour such as increased anxiety, anhedonia, sleep disturbance,

decreased appetite and increases in the ACTH and glucocorticoids responses to a variety of stressors are observed in infants that had experienced disruption in maternal care in the first 14 postnatal days (PND) (Heim *et al*, 2004). In addition to this, neurobiological studies of early life stress in rats observed marked CNS changes such as an increase in CRH activity, decrease in glucocorticoid receptor density in the hippocampus and prefrontal cortex (Heim *et al*, 2004) or reduced astroglial density in the limbic system of ED rats (Leventopoulos et al., 2007). Therefore, early life stress results in various neurobiological changes such as alterations in the HPA axis system, the SAM system, the serotonergic and mesolimbic dopamine pathways (Heim *et al*, 2004; Leventopoulos *et al*, 2009; Rüedi-Bettschen *et al*, 2005). It is of prime importance to understand the mechanisms, which activate stressful events and which serve as a link between early life stress and brain development (Brunson *et al*, 2001). Studies carried out on early deprived rodents reveal multiple changes in various neurocircuits involved in neuroendocrine and behavioural responses to stress, which may cause an increased risk for depressive disorders in humans (Heim *et al*, 2004).

Extrapolating to the human condition, healthy physical and mental development is of prime importance during the childhood and adolescence stage for the betterment of adulthood life but stress related deficit is common during these phases. A large-scale study carried out by Cohen *et al* (2006b) on early life stress (ELS) (n=1659) found that more than one-third of the participants suffered from adverse childhood events mostly were divorce (22%), family conflicts (20%) and social abuse (17%). ELS leads to stress response system dysfunction and reduced immune function which may have prolonged effects continuing into adulthood (Graham et al, 2006).

A study reported significant reduction in the anterior cingulate cortex and caudate nucleus volume (brain regions involved in emotional and cognitive behaviour apart from movement control) in healthy adults who had a previous history of adverse childhood events (Cohen *et al*, 2006a). Another study found significant left hippocampal volume reduction in adult women who suffered from childhood sexual abuse (Sapolsky, 2000). Teicher *et al* (2004) reported a 17% decrease in the corpus callosum volume in socially abused and neglected children compared to non-exposed group. Studies have found links between ELS and cognitive function; with ELS effects on mental functions, information processing, memory, intelligence, verbal abilities, low IQ's (Goethals *et al*, 2003). ELS may play a role in the alterations of white matter pathways in the brain also indicates the vulnerability of the brain during these critical periods of development (Seckfort *et al*, 2008).

1.2.2 Early life environmental manipulation

As mentioned earlier, the insights of behavioural and physiological mechanism involved in any neurobiological disease are identified using animal models (McEwen, 2007). In rodents, early life maternal care is important factor which determines long term emotional and stress hormone reactivity and increase in both leads to earlier cognitive decline and shorter life span (Francis et al., 1999).

Strong mother – infant interaction, more licking and grooming, produces offspring which is more exploratory of novel environment and less emotionally reactive known as

a *neophilic* animal. This type of strong maternal behaviour leads to decreased levels of glucocorticoid response in novel situations. Poor mother – infant interaction, less licking and grooming, results in offspring which is less exploratory of novel environment and with increased emotional and HPA reactivity, known as a *neophobic* animal (Meaney et al., 1994).

The neonatal handling involves disruption in maternal care, short term (decreased HPA reactivity) and long term (increased HPA reactivity) separation of pups from their mothers whereas, enriched environment during the peripubertal period overcomes the neurobiological deficits resulted from low maternal care (Levine et al., 1955).

The influence of the nature and nurture is an important factor for the social well being of an individual. The effects of early life manipulation of the infant's social environment can be studied at different time points. The phenotypes that are developed are associated with depression like neurobiological changes and behaviour observed in rodents and which are reversible with anti-depressant treatment (Pryce et al., 2005; Leventopoulos et al., 2007). These depression-like phenotypes emerge into behavioural, physiological and neurobiological test paradigms (Figure 1.3) which can be quantified may provide insights into understanding the mechanisms involved in depression.

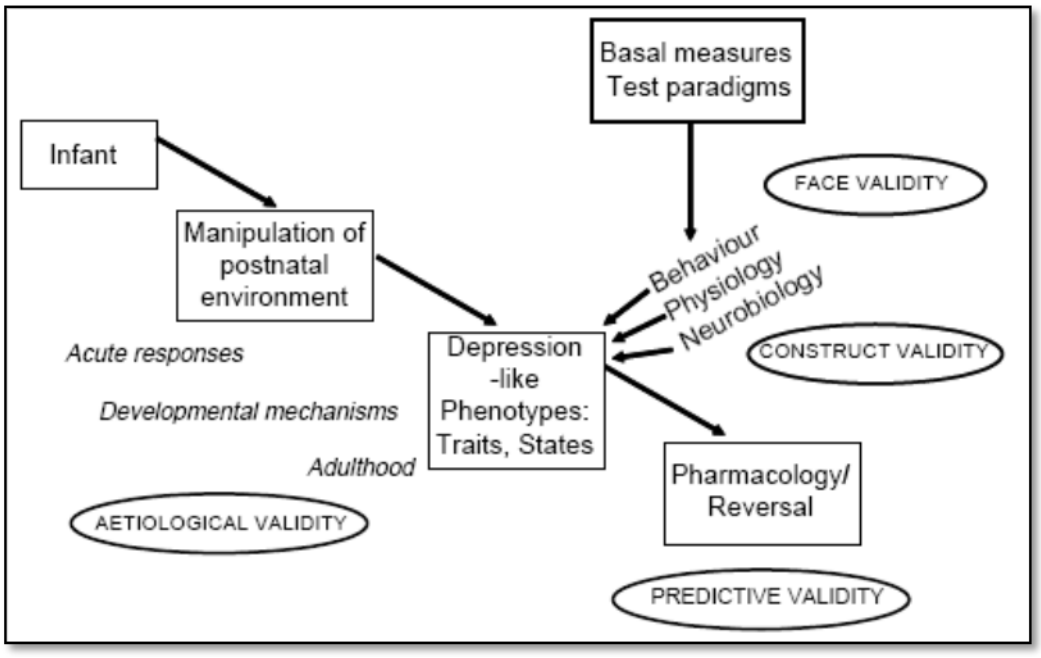

Figure 1.3 Early life manipulation yields animal models of stress and depression.

(Source: Pryce *et al*., 2005)

The above diagram represents various stages, mechanisms and measures involved in the development of the animal models of depression and stress.

1.2.3 Types of early life environmental manipulation (postnatal)

Postnatal manipulations during the early life developmental period result in loss of homeostasis which can cause long term changes (Pryce *et* al, 2005; Sapolsky, 2004). Such postnatal manipulations were characterised long ago and have been studied experimentally for nearly 50 years. The work in the field is pioneered by Seymour Levine and Victor Denenberg (Levine *et al*, 1955; Denenberg et al., 1963).

The postnatal manipulations in rodents and primates which may disrupt the homeostatic relationship between mother and infant are achieved mainly by early-handling and early-deprivation (Pryce *et al*, 2005).

1.2.3.1 Early-Handling (EH)

In EH manipulation mother and infants are exposed daily to human physical contact and are exposed for 15 min to a different physical environment. Mother and infants are always separated whereas infants are separated from dam either as an intact litter or as individual pups (Pryce and Feldon., 2003). EH of rats leads to adult offspring that are less anxious and fearful when exposed to environmental challenge (Levine et al., 1955; Pryce et al., 2001). EH rats attract increased maternal attention in the form of licking and nursing in comparison to non-handled rats (NH) (Liu et al., 1997). Reduced ACTH and GC responses are also observed together with enhanced cognitive abilities in adulthood (Pryce et al., 2001a, b).

1.2.3.2 Early-Deprivation (ED)

In ED rats, mother and infants are exposed daily to human physical contact and 0.5–6 hours of daily separation of individual infants from littermates and from the mother, in a different physical environment during the PND 1-14 is performed (Pryce and Feldon., 2003). The mother remains in the home cage. The procedure is performed at the ambient colony room temperature (Pryce et al., 2005). (Figure 1.5)

1.2.3.3 Non-Handling (NH)

To control for EH and ED, NH rats are used where mother and infants are not exposed to human physical contact and exposed to only minimal distal human disturbance, e.g. room entry restricted to one person only during the postnatal period of life (Pryce et al., 2005). Studies have observed that NH rats move less in the open field test and are characterised by increased acoustic startle and corticosterone response (Pryce et al., 2001). In later stages of adult life, NH rats seem to show increased levels of basal hypothalamic CRF mRNA and increased CRF in the median eminence (Plotsky and Meaney, 1993).

Early maternal deprivation in rats is a model which replicates the relation between early life stress and depression and reflects similar human traits such as neglect like behaviour and reduced motivation to obtain the reward (measured by sucrose test) (Pryce *et al*, 2005; Rüedi-Bettschen *et al*, 2005). Early deprived rats develop marked changes in behaviour such as increased anxiety, anhedonia, sleep disturbance, decreased appetite and also shows three – fold increase in the ACTH and glucocorticoids responses to a variety of stressors when experienced separation from mother for 180 min in the first 14 postnatal days (PND) (Heim *et al*, 2004).

Behaviourally, ED rats are more active in the open field with a reduced acoustic startle reflex and show enhanced two way active avoidance in comparison to NH rats (Rüedi-Bettschen et al., 2005).

An ED procedure established at the Swiss Federal Institute of Technology in Zurich, Switzerland consists of isolation of rat pups from both the dam and littermates for 4

hours/day on 1-14 postnatal days (PND). The pups are placed in open plastic compartments on a thin layer of saw dust, at ambient room temperature during and the dark phase of a reversed light-dark cycle. Absorbent paper is placed on the bottom of the cage for urine and feces. The cage is placed in an isolation chamber that is illuminated with a 4-W bulb. A small video camera placed above the cage which allows for the behaviour to be observed and recorded (Figure 1.4).

Figure 1.4 Early deprivation procedure where rat pups are placed in separate compartments. (Source: Pryce *et al*., 2005)

Picture representing early deprivation procedure carried out at ETH, Zurich laboratory, where rat pups are placed in open plastic compartments on a thin layer of sawdust.

1.2.4 Early responses to postnatal manipulations

As mentioned above, the effect of stress depends on the duration and the level of stress, which further results in an acute or chronic response depending on stress exposure. The neurochemical changes occurring in the phase of postnatal deprivation can lead to

detrimental effects causing morphological and functional changes in neurones and astroglia (Watanabe et al., 1992; McEwen, 2005). McCormick *et al* (1998) suggest that repeated daily separation of pups from the mother can lead to an increase in stress responses which may result in higher GC levels causing disruption of memory and learning processes mediated by the hippocampus (McCormick *et al*, 1998; Zhang *et al*, 2002). Levine *et al* (2001) conclude that isolation of rat pups from their mothers for eight hours can cause acute stress and result in increased levels of ACTH and glucocorticoids following an increase in HPA axis activity. A study carried out on daily maternally deprived rats on 2-14 PND then followed by a single acute 3 hour separation have demonstrated a significant increase in the expression of BDNF mRNA in the hippocampus and prefrontal cortex (Roceri et al, 2004). Increased serotonin activity and GC mRNA expression in the hippocampus is observed in early handled rats (Meaney et al., 2000). Maternal separation (6 hours/day – PND 2-14) affecting the early rearing environment in male rats showed impairment in the programming of fear conditioning and extinction (Stevenson et al., 2009). Slotten et al. (2006) found long-term gender dependent effects on the behaviour and HPA axis function in rats exposed to maternal separation (PND 3-15, 3 hours/day). These early responses are of prime importance to understand the mechanisms which lead to long term effects of early life stress and pathophysiology of depression.

1.2.5 Long-term responses to postnatal manipulations

Research to date suggests that long term effects of postnatal manipulations cause varied changes in the neurobiological and neuroendocrinological systems. These early life

alterations result in an overactivity of the HPA axis with increased levels of CRH and corticosterone, which may have a detrimental impact on hippocampal neurones contributing to the onset and pathophysiology of depression (Nemeroff, 1998; Swaab *et al*, 2005). Hypercortisolaemia (high GC concentrations in blood) (Fuchs and Flugge, 1998) may lead to glial and neuronal cell death.

The postnatal manipulations lead to depression-like behavioural phenotypes in rats and have long-term outcomes in behavioural and endocrine responses (e.g. Pryce *et al*, 2005). These effects can be investigated by using tests to monitor anhedonia and the coping ability of animals. ED rats exhibited a significant reduction in the motivation to obtain reward in the progressive ratio schedule test compared to NH (Pryce *et al*, 2005). In-house studies have demonstrated that stress hyper-responsive Fischer rats exposed to early life deprivation developed into adults with reduced astroglial GFAP-immunoreactivity in the limbic regions of the brain (Leventopoulos *et al*, 2007). In normal responsive Wistar rats, early deprivation leads to decrease in the 5HT1A receptor binding (Leventopoulos et al., 2009). Evidence of this kind suggests that early adverse life experience leads to long-term neuro-behavioural abnormalities.

1.3 Prenatal Stress

Prenatal period, which lasts from fertilisation to birth, is complex and interesting as the stress system of the neonate during this time is dependent, influenced and closely linked with the stress system of the mother. It is during this time the central components of the HPA axis emerge and begin to function (Challis et al., 2001). In rodents, at gestational day 18, the foetal HPA axis responds to maternal stress with elevated ACTH levels (Ohkawa et al., 1991). The fetal HPA axis can also be stimulated by the maternal CRH originating from the placenta (Weinstock, 2005).

Excess glucocorticoids during this development phase are detrimental. Foetal glucocorticoids produced are mainly of maternal origin. The placenta acts as the protective element against foetal exposure to maternal glucocorticoids by high activity of enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which converts corticosteroids to inactive 11-keto forms. Excessive or long lasting maternal stress leads to deficiency of the 11β-HSD2 enzyme further leading to damaging effects on foetal development (Figure 1.5) (Seckl and Holmes, 2007). In humans, prenatal stress exposure is linked to new born infants with low birth weight further developing neurological disorders (Van den Bergh et al., 2005; Schmidt, 2010).

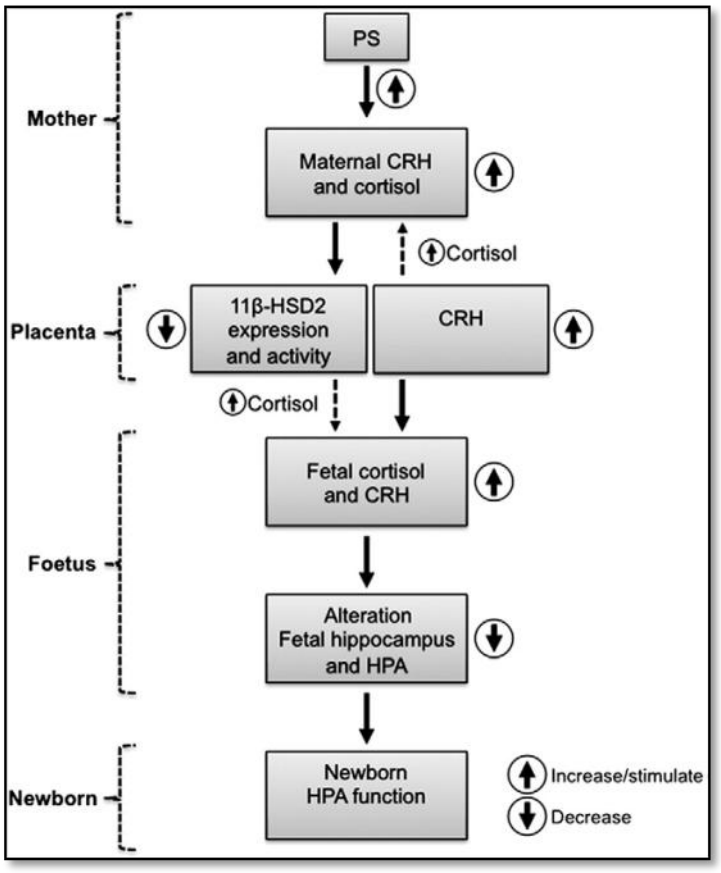

Figure 1.5 - Prenatal stress effects on the foetal HPA axis functioning

(Source: Charil et al., 2010)

(*PS – prenatal stress, CRH – corticotropin releasing hormone, 11βHSD2 – hydroxysteroid dehydrogenase, HPA – hypothalamus pituitary adrenal axis*)

The above pathway details the activation of foetal HPA axis via the maternal CRH and cortisol release affecting foetal development.

In comparison to humans, rodent's prenatal period corresponds to the early prenatal period in humans (Figure 1.6).

Prenatal stress is related to cognitive, physical behavioural and emotional disorders in later life. In humans, it is associated with various psychosocial problems during childhood and adulthood development (King and Laplante., 2005; Charil et al., 2010). Prenatal stress due to natural calamity is also linked with developing depression and schizophrenia in adulthood (Watson et al., 1999; Kinney, 2001).

Preclinical studies in animals have observed that prenatal stress produces specific behavioural changes such as non-directed locomotor activity, anxiety, and decrease in memory and learning tasks (Coe et al., 2003; Wu et al., 2007). These cognitive, behavioural and psychosocial deficits are possibly caused by maternal stress which shows damaging effects on the offspring's brain (Anderson et al., 1985; Coe et al., 2003).

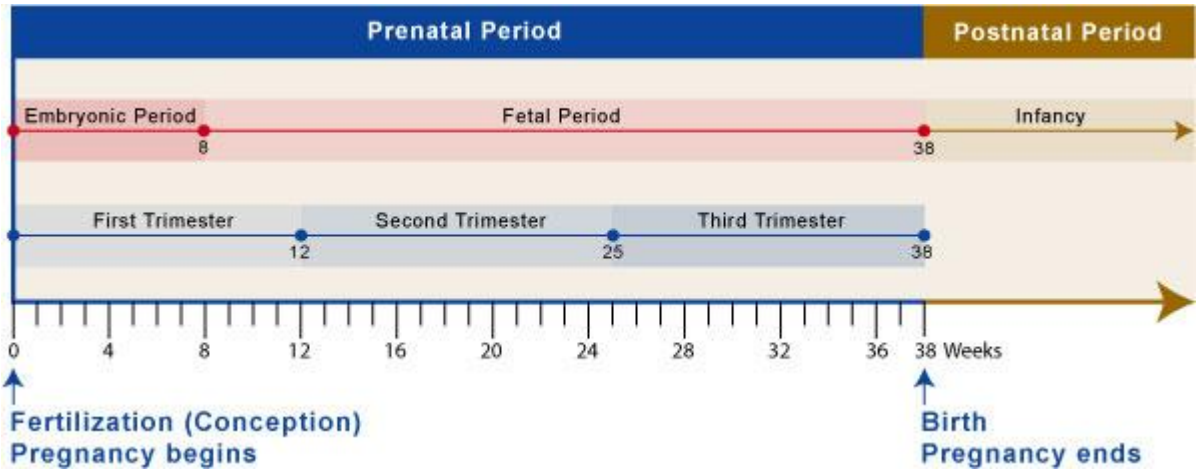

Figure 1.6 - Human prenatal period timeline.

(Source:http://www.ehd.org/dev_article_intro.php)

Red and blue line indicates different stages and time periods involved during pregnancy and early human development.

In humans, monkeys and rodents, glucocorticoids play a key role in the development of the foetus, which is why its levels are high during pregnancy (Seckl and Holmes, 2007). However, in the event of maternal stressful condition GC levels reach abnormally high levels which may have potential effect on the foetal growth. Animal studies have found alterations in the hippocampal volume and neuronal development in the brain due to high corticosteroid exposure during the prenatal period (Uno et al., 1994; Avishai-Eliner et al., 2002; Coe et al., 2003).

In human pregnancy, maternal HPA axis regulates the placental CRH activity which in turn influences the foetal hippocampal development (Wadhwa et al., 1998). Prenatal stress increases the hypothalamic CRH release in the bloodstream. Suppression of hypothalamic CRH takes place while placental CRH is increased by glucocorticoids leading to higher foetal plasma CRH levels (Sandman et al., 1999). Activation of CRH receptors results in altered foetal HPA axis function which has a negative impact on the postnatal development of the new born (Avishai-Eliner et al., 2002).

1.3.1 Effects of prenatal stress on brain regions

The key region affected by prenatal stress is the hippocampus which develops primarily during the foetal period (Rice and Barone, 2000). Hippocampus is the region where neuronal cell proliferation takes place and is actively involved in learning and memory function. Early studies carried out on non-human primates suggest hippocampus as the prime target of both pre and postnatal stress because corticosteroid administration alters normal hippocampal functioning (Uno et al., 1994). Hippocampal volume loss and a

decrease in neurogenesis in the dentate gyrus region are observed in primates exposed to prenatal stress (Coe et al., 2003).

Amygdala, a brain region involved in emotional regulation, appears to be also affected by prenatal stress. Thus offspring of Sprague Dawley rats exposed to a novel environment following saline injection to pregnant dams during gestational days 14 and 21 have shown reduction in amygdala volume and a decrease in the number of neuronal and glial cells (Kraszpulski et al., 2006).

The other effects of prenatal stress reported in experimental animals are, a decrease in the size of the corpus callosum (Coe et al., 2002), morphological changes in neurones and glial cells in the cerebral cortex region (Poland et al., 1999), loss of interneuronal connectivity and decreased granular cell nuclei volume in the cerebellum (Ulupinar and Yucel, 2005) and in hypothalamus differences in the sexually dimorphic nucleus of preoptic area size (Anderson et al., 1985).

1.3.2 Differences and limitation to prenatal stress in animal studies

Even though the rat lifespan is 3 years, it does not indicate that it lives a miniature human lifetime within those 3 years life time (Quinn, 2005). The developmental stages such as infancy, weaning, puberty, adulthood, reproductive senescence, post senescence are similar in both humans and rats but their timings are different. The approximation may be considered during the periods of development but straight age translation could not be reasonable between humans and rodents (Quinn, 2005, Clancy et al., 2001).

The central nervous system develops in a time-dependent manner. Duration and time of the neurodevelopmental events and the ratio of specific brain regional volume to whole brain volume is different in different species (Rilling and Insel, 1998; Charil et al., 2010). In humans the brain maturity level is different compared to rodents at birth. Rodents are born with an immature brain which develops mostly during the postnatal period whereas human brain development starts prenatally (Rice and Barone, 2000).

In case of prenatal stress studies various factors can affect the outcome, which includes differences in species with varying gestational periods (in rats 21.5days, monkeys 165 days and humans 270 days), type of stressor applied, timings of stress administration and postnatal assessment, as well as effects of sex, maternal care and epigenetics.

1.4 Genetics and early life experiences

As mentioned earlier, early life experiences or environment may have an enduring effect on the CNS as infant brain rapidly changes during post-natal days. Neurodevelopment of infants depends on the maternal care in the form of feeding and warmth. In order to understand how the brain is shaped by these experiences, studies have been conducted on the neurodevelopment of rodent pups who receive either low or high levels of maternal care (Liu *et al*, 1997). Like human infants, newborn rodent pups, need to be fed, kept warm and stimulated in order to grow. This stimulation is provided by licking and grooming (LG). However, not all mothers provide the same level of maternal care to their offspring. Individuals who receive low levels of LG are found to have fewer

glucocorticoid receptors within the hippocampus as well as altered dopamine, serotonin and GABA neurochemical pathways in the brain. These neural changes emerge early in infancy and are sustained into adulthood with consequences for behaviour across the lifespan (Liu *et al*, 1997).

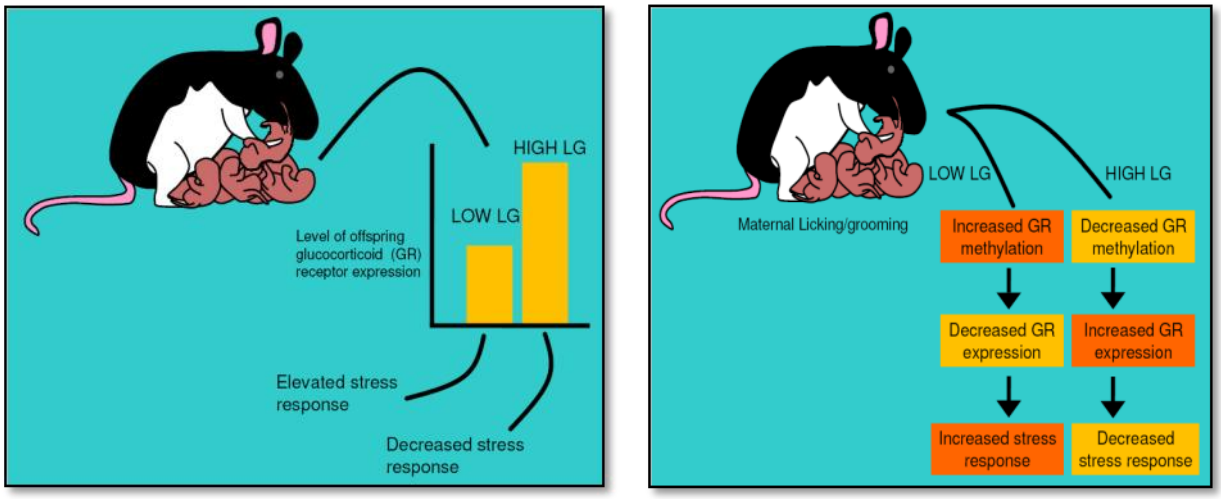

Figure 1.7 - Level of offspring glucocorticoids receptor expression in relation to maternal licking and grooming and stress responses. (Source: Champagne *et al*., 2003)

(*LG – licking and grooming, GR – glucocorticoid receptors*)

The above figure indicates low level of LG results into increased stress response due to high GR methylation and high level of LG causes reduced stress response due to high GR expression resulting from lower GR methylation.

The stable increase in the activity of ERα gene (estrogen receptor) allows the "inheritance" of maternal care. This raises the argument of how changes to a gene's

activity can persist long after the experience of maternal care. Levels of gene expression are dependent on how accessible the sequence of DNA is to factors which initiate the transcription process. These factors can be blocked by a stable and heritable chemical modification known as DNA methylation. Once DNA is methylated it is no longer available to transcription factors and gene expression; it is thus "silenced". DNA methylation is referred to as "epigenetic" because this modification alters the activity of genes without altering the sequence of their DNA (Champagne *et al*., 2003).

Studies of maternal behaviour in the rat suggest that mothering is transmitted "epigenetically" from mother to daughter through levels of methylation of the ERα gene promotor. Studies have also shown that the experience of low levels of maternal care in infancy can lead to increased levels of DNA methylation within the promotor region of the ERα gene leading to reduced levels of receptors in adulthood. In this way, the quality of mother-infant interactions can act as an "on/off" switch to gene expression with consequences for neurobiology and behaviour (Champagne *et al*., 2003).

While the brain is likely to be particularly sensitive to experiences occurring early in development, there is a high degree of plasticity in the brain beyond infancy. Maternal and social behaviours can be influenced by the quality of the juvenile environment and prolonged exposure to stress is effective at shifting patterns of behaviour across the lifespan (Hane *et al*., 2006). Though it is not yet known how these changes occur, it is possible that these environmental experiences turn genes "on" or "off" through epigenetic pathways (Champagne *et al*., 2003). In gaining a better understanding of how

the environment can shape gene expression and the brain we may be able to develop new strategies for intervention in humans to reverse the effects of early life adversity.

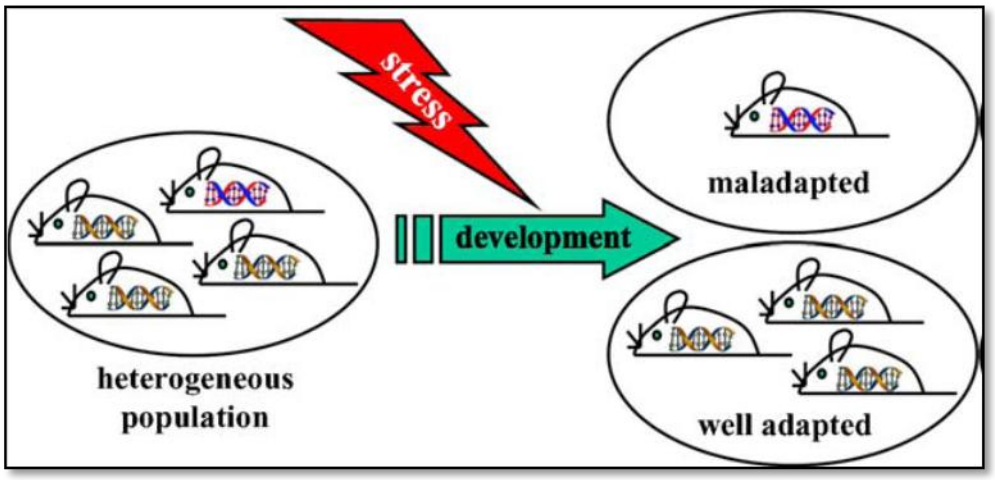

Figure 1.8 Resilience and susceptibility to early life stress (Source: Schmidt, 2010)
The applied stressor during the developmental phase results into either adaptive (beneficial) or maladaptive (detrimental) offsprings.

Preclinical research suggests the fact being overlooked is that the majority of the individuals are resilient to early life stress. It is the genetic makeup (epigenetic) of the individual which decides whether stress exposure during prenatal or postnatal development leads to maladapted with high risk factor of disease later in life or well adapted with no vulnerabilities (Fig 1.8). Gene-environment interactions play an important role in early life stress. Interestingly, individuals with short allele of the serotonin transporter gene (5-HTT) possess high chances of depression later in life when

they were maltreated during childhood whereas individuals with long allele of 5-HTT gene are protected (Capsi et al., 2003). 5-HTT gene depleted mice show susceptibility to early life stress (Carola et al., 2008) which supports a role of genetics in depression.

1.5 Depression and its links with early life experiences

Depression is a common psychiatric disorder which affects one in six people and leads to suicide in approximately 10% sufferers. Depression is expected to become the second leading cause of disease burden by 2020 (WHO 2007). The brain mechanisms that lead to depression have not been fully explained despite years of clinical and preclinical studies, although chronic stress is considered to be a risk factor.

Published research suggests a role of multiple neuro-endocrine, neuro-chemical and neuro-anatomical brain changes in major depressive disorder (e.g. Heim *et al*, 2004). Considerable evidence also indicates involvement of neuronal and glial changes in certain regions of the brain which are responsible for the modulation of stress and emotion (Duman, 2002). Ethical restrictions which limit the clinical studies in depressive patients confine the knowledge of neurobiological processes and mechanisms involved in depression (Fuchs *et al*, 2004). To overcome this, rodents and primates have been used in experiments designed to investigate the neurobiology of depression and depression-like conditions.

Alterations and modification of neuronal circuits, cell morphology and synaptic connections due to external or internal stimuli are known as neuroplasticity.

Neuroplasticity is the ability of the brain to adapt functionally to various challenges occurring due to external environment (Fuchs and Flugge, 1998; McEwen, 2000). Capacity of neural systems, brain pathways, neurons, synapses, and receptors play an important role in this process (Fuchs *et al*, 2004; Zilles, 1992). Neuroplasticity occurring in response to environment is of prime importance for brain function in health and disease, but it is not always beneficial. Alterations in the structure and function of the brain in patients with depressive disorder illustrate deleterious effects of neuroplasticity (e.g. Duman, 2002; Fuchs *et al*, 2004) and they will be addressed below.

Mechanisms behind neuroplastic changes occuring in the brain which mediate depression, leading to return of depressive symptoms in successfully treated patients remains unclear. Various neuroimaging studies in patients suffering from depressive disorder and preclinical studies in animal models of depression show involvement of certain brain areas (involved in memory and emotion) such as hippocampus, amygdala and prefrontal cortex, are thought to play a role in developing symptoms of depression (Bremner, 2005).

One of the important regions of the brain affected by depressive disorders is the hippocampus. Hippocampus is the part of brain which deals with emotional processing (ventral hippocampus) and learning and memory processing (dorsal hippocampus) (Fuchs and Flugge, 1998; McEwen, 2000). Both clinical and experimental research suggests a role of the hippocampus in major depressive disorder (e.g. McEwen, 2001). Structural neuroplasticity in the form of remodeling of dendrites takes place in the CA3 region of the hippocampus (Duman, 2002). Dendritic remodeling could be the basis of

neuroplasticity effects and the reason of a reduction in the hippocampal volume in depression (Duman, 2002, 2004; Fuchs *et al*, 2004). Indeed, neuroimaging studies by means of MRI show a reduction in the hippocampal volume in patients suffering from long term depression (Coe *et al*, 2003; Nemeroff, 1998). Reduced volume of right hippocampus and left amygdala have been observed in patients suffering from bipolar depression (Bremner *et al*, 2000).

Stress-induced hippocampal plasticity reported in animal studies as being associated with cortisol has given new insights into the relationship between hippocampus, memory and cortisol in patients suffering from depressive disorders. Hypercortisolaemia in patients with Cushing's syndrome is linked with a reduction in the hippocampal volume similar to depression (Starkman et al, 1992). Enlarged pituitary and adrenal gland, increase in the corticotropin-releasing factor (CRF) and non-suppression in the dexamethasone suppression test leading to high levels of cortisol in the periphery are observed in the depressed patients (Nemeroff et al, 1984 and 1992).

Hippocampal plasticity is a well-replicated finding in depression. Hippocampal volume shrinkage and reduction in the hippocampal neuronal size are the consistent effects of stress on hippocampus. Hippocampus holds a key function possessing an inhibitory effect on the HPA axis through which CRF release from the hypothalamus could be blocked. In depression, hippocampal damage causes loss of negative feedback inhibition leading to excessive release of cortisol contributing to hypercortisolaemia and detrimental effects to normal brain functioning (Jacobson et al, 1991).

The amygdala plays an important role in the emotional memory formation and emotion regulation. Studies also suggest its role in pathophysiology of mood disorders (Fuchs *et al*, 2004). MRI studies carried out on female patients with depression showed a reduction in amygdala core (Sheline et al, 1998) whilst another study on men and women showed a larger total amygdala volume in depressed patients (Bremner *et al*, 2000). A post-mortem histopathological study showed a decrease in the number of glial cells in the amygdala in major depressive disorder (MDD) patients with no change in neuronal numbers (Bowley *et al*, 2002). Another study with a stereological approach carried out on MDD patients showed that a decrease in glial cells in the amygdala primarily was due to oligodendrocyte reduction (Hamidi et al, 2004). The reported glial changes are likely to be causally linked with the volume reduction in the amygdala.

Prefrontal cortex (PFC) dysfunction plays an important role in the aetiology of major depression and has connections with hippocampus (Nemeroff, 1998). Recent findings also suggest that dendritic alterations observed in PFC and hippocampus are due to high levels of glucocorticoids, which indicates that these biochemical mediators of stress play a role in neuroplasticity (Fuchs *et al*, 2004).

Volumetric brain imaging carried out by Sheline et al. has found a relationship between disease chronicity and hippocampal volume loss. Protective effects of antidepressants have been observed in patients who were previously untreated for depression showing an inverse correlation between the hippocampal volume and the duration of antidepressant treatment (Sheline et al, 2003). Hippocampal volume loss in patients with multiple episodes of depression but not new-onset depression was reported

(MacQueen et al, 2003). No changes in the hippocampus volume have been observed in young depressed patients (Rusch et al, 2001). These studies suggest that duration or chronicity of illness plays a key role in determining hippocampal atrophy in depression.

It is estimated that a 40-50% chance of having depression is genetic, although the specific genes have not been identified (Nestler *et al*, 2002). Genomic and epigenetic changes may occur due to maternal care disruption which influences the development of the offspring (Weaver *et al*, 2004). Environmental factors such as parental death, social neglect, sexual abuse, or chronic illness which occurs in early life can lead to depression in later life (Agid *et al*, 2000). Early life stress (ELS) is considered to be a risk factor of depression (e.g. Agid *et al*, 2000; Heim and Nemeroff, 2001). In support of the latter is a clinical study involving 2000 women revealed that those with a history of sexual abuse in childhood developed most of the symptoms of depression and had more attempts of suicide than women without any history of childhood sexual abuse (McCauley *et al*, 1997). There is consensus that a depressive disorder is the outcome of the ongoing stress in conjugation with multiple other psycho-physiological effects (Agid *et al*, 2000; Heim and Nemeroff, 2001).

1.6 Glucocorticoids and its links with early life experiences

Glucocorticoids, cortisol (in humans) and corticosterone (in rodents) provide one of the most powerful endogenous feedback systems, which exert negative regulation that sets the basis for stress related activity of the HPA axis (de Kloet *et al*, 1999; Heim *et al*,

2004). Glucocorticoids (GC) control the excitability of neuronal networks, which support the learning and memory processes (de Kloet *et al*, 1999).

As mentioned earlier, GCs bind to two different types of receptors type 1-mineralocorticoid and type 2-glucocorticoid receptors. MRs are found majorly in hippocampus whereas GRs are concentrated in the entire brain (Reul and de Kloet, 1985). These receptors show different affinities towards cortisol and other glucocorticoids such as dexamethasone, prednisone. GR shows less affinity for cortisol which makes it more critical during stress response when plasma cortisol levels increases (Meaney et al, 1988). Glucocorticoid receptors (GRs) are highly expressed in various brain regions, such as hippocampus, amygdala and prefrontal cortex (limbic system of the brain - involved in the feedback regulation of the HPA axis) (Herman *et al*, 2005). The GRs are found abundant in hippocampus which actively responds to the GC release from adrenal glands and stimulates the negative feedback mechanism of HPA. As a result, inhibition of CRH and further ACTH release occur via GC-dependent negative feedback mechanism of HPA axis (De Kloet *et al*, 1999; Herman *et al*, 2005).

The release of GC is known to cause several morphological and physiological alterations in the central nervous system (CNS) (McEwen et al, 1986). High levels of glucocorticoids have a detrimental impact on the normal functions of brain, which leads to an impairment in the processes of memory and learning along with hippocampal dendritic atrophy (Colla *et al*, 2007; Fuchs and Flugge, 1998). The limbic system is highly sensitive to endogenous glucocorticoids during the period of brain development (Matthews, 2000).

Glucocorticoids regulate the glial fibrillary acidic protein (GFAP) gene expression in non-neuronal cells called astroglia; reduced GFAP gene expression correlates with the degree of astroglial responses in the adult brain (Nichols et al, 2005) and shows a reduction in the delayed astrocytic maturation in developing brain (Huang *et al*, 2001). The glucocorticoid receptor expression levels in the hippocampus are regulated by early life experiences across life span via epigenetic processes (natural variations in maternal care influence HPA axis stress reactivity in the offspring via long term changes in tissue-specific gene expression) which suggests a link between early deprivation (ED) and GFAP levels which were examined in a rat model (Weaver *et al*, 2004; Leventopoulos et al., 2007). Research suggests that exposure of neonatal rats to high levels of glucocorticoids leads to a reduction in the brain weight (Howard and Benjamin, 1975), reduction of dendritic spines and neuronal atrophy (Cotterrell *et al*, 1972). High level of glucocorticoids causes alteration in social behaviour and learning processes (Sousa *et al*, 2000). Glucocorticoids released as a response to stress down regulate the brain derived neurotrophic factor (BDNF) which is important for neurogenesis and synaptogenesis (elements of neuroplasticity) (Russo-Neustadt, 2003). Wistar rats exposed to glucocorticoids show a reduction in the dendritic length and density and CA3 apical dendritic atrophy in comparison with control rats (Sousa *et al*, 2000). There is also evidence of cell loss and cell atrophy in prefrontal cortex (PFC) which indicates that glucocorticoids target PFC which contains high levels of glucocorticoid receptors (Heim *et al*, 2004; Stark *et al*, 2006). Overall, there is growing consensus that glucocorticoids as mediators of stress affect brain plasticity in multiple ways.

1.7 Glial cells

Cells known as glia (Greek for "glue") were long thought to provide support to nerve cells while neurones were considered as the main functional unit of the brain. However, more recent research has revealed that glial cells not only play a supportive role but are an active participant in brain function which includes brain development, formation and function of synapses, immune response and brain homeostasis (Kurosinki and Goetz, 2002; Garcia-Segura and McCarty, 2004; Jauregui-Huerta et al., 2010).

1.7.1 Types of glial cells

Glial cells are mainly divided into two types in the CNS, macroglia and microglia. Based on their phenotype and function these two types are further divided into various subtypes of cells. Microglia, also known as resting microglia in the normal uninjured brain, are very active and versatile cells. They are considered as immune cells of the CNS because they are the early detectors of the brain injury and become activated in response to damage by altering their morphology and expression pattern. Even in resting stage they serve as very active biochemical sensors. Thus, microglial cells play important role in prevention from brain damage and support in maintaining brain homeostasis. (Kim and de Vellis, 2005; Hanisch and Kettenmann, 2007; Napoli and Neumann, 2009). Macroglia include different sub cell types - astroglia, oligodendroglia, and ependymal and Schwann cells (in the peripheral nervous system). Astroglial roles are discussed below. Oligodendroglia and Schwann cells play a key role in myelination of axons in the central and peripheral nervous system, respectively, and ependymal cells

play a protective role by lining cavities of CNS and forms wall of the ventricles in brain and central canal in the spinal cord. (Jauregui-Huerta et al., 2010).

1.7.2 Astroglia

Astroglial cells are the most abundant cells and involved in variety of roles in the brain. Astroglial cells include different types of cells - astrocytes, radial glia, Bergmann cells, Muller cells, pituicytes and tanycytes.

Astroglial functions include regulation of synaptogenesis, support of neurogenesis and gliogenesis, guidance in neuronal migration, regulation of cerebral microcirculation, provision of energy substrates for neurons, regulation of extracellular ion concentrations and extracellular pH, modulation of neurotransmitter signalling and recycling, regulation of the brain water homeostasis, modulation of synaptic transmission, integration and regulation of synaptic networks, neuroplasticity and modulation of neuroendocrine functions (Blankenfeld and Kettenmann 1992; Cohen et al 1999; Hirst et al 1998; Kimelberg and Katz 1985; Russ et al 1996; Schousboe and Westergaard 1995; Garcia Segura and McCarthy, 2004). Astroglia are involved in various important functions such as providing neuronal cells with pyruvate, lactate, neurotrophic factors and energy storage (Dringen et al., 1993), promote synaptic formation of neuronal cells from stem cells (Song et al., 2002) and metabolism of glutathione and reactive oxygen species (Wilson, 1997; Sharma et al., 2007).

Recent studies have suggested an involvement of astroglial cells in the pathology of stress and glucocorticoid overproduction. Reductions in the astroglia density and number

are linked with the region-specific volume changes which are observed in stress related pathologies (Rajkowska and Miguel-Hidalgo, 2007; Czeh et al., 2007). Synthetic glucocorticoid – dexamethasone is found to block the astrogliogenesis process observed in neural precursor cells (Sabolek et al., 2006). However, these findings do suggest an unknown role of astroglial cells in response to stress and glucocorticoids which needs to be further researched in order to fully understand the neuroprotective role of glial cells.

Astroglial plasticity in the form of morphological changes have been reported in response to stress. Studies have shown long lasting astroglial reactions and decreased dendritic arborisation in response to prenatal stress (Barros et al., 2006) and increase in the glial fibrillary acidic protein (GFAP) immunoreactivity in the hippocampal CA1 region and striatum following repeated immobilization stress (Kwon et al., 2008).

Reduced GFAP immuno-reactive (ir) cells and cell proliferation decrease was seen in the prefrontal cortex and dentate gyrus in rats exposed to chronic social stress (Czeh et al., 2007; Fuchs et al., 2004). Similar changes in the GFAP-ir cell have been observed in the amygdala, hippocampus and prefrontal cortex of the rats with depressive like behaviour (Gosselin et al., 2009). Hippocampal GFAP –ir reductions have also been reported in maternally deprived stress-hyperresponsive Fischer rats (Leventopoulos et al., 2007). Reduced GFAP expression due to chronic unpredictable mild stress, which was partially rescued by hippocampal brain derived neurotrophic factor (BDNF) administration, provides insights into the glial physiology as a therapeutic target in depression and other stress-related conditions (Ye et al., 2011).

1.7.3 Glial Fibrillary Acidic Protein (GFAP)

Glial fibrillary acidic protein (GFAP) is the main intermediate filament protein in mature astrocytes, in addition to vimentin, nestin and synemin, but also an important component of the cytoskeleton in astrocytes during development, aging and in neurodegenerative diseases. The intermediate filament network is a key component of the cells cytoskeleton, integrity and resilience. There are different isoforms and splice variants of GFAP – GFAP α, β, γ, δ and κ, but the differential roles and distribution of these isoforms is not yet clear (Andreiuolo et al., 2009; Blechingberg et al., 2007; Roelofs et al., 2005).

Classically, GFAP is a marker for astrocytes, known to be induced upon brain damage or during CNS degeneration, and to be more highly expressed in the aged brain. GFAP has become a prototypical marker for immunohistochemical identification of astroglial cells (Sonfroniew and Vinters, 2010). Initially, GFAP was identified in the brains of patients with multiple sclerosis, in which GFAP was purified from a large multiple sclerosis plaque consisting primarily of fibrous astrocytes and demyelinated axons (Eng et al., 1971).

GFAP belongs to the family of intermediate filaments that, along with microtubules and microfilaments, make up the cytoskeleton of most eukaryotic cells. Major developments in astroglial biology and the discovery of novel intermediate filament functions have shown interest in the function of GFAP (Eng et al., 2000).

1.7.4 Effect of stress on astroglia

Effects of long term stress and depression on neurones, mostly in the limbic system and prefrontal cortex have been demonstrated by a number of studies (e.g. McEwen, 2001, 2005). However, there is growing evidence that glia are also implicated in responses to stress and depression, and responsible for depression related brain remodeling (Harrison, 2002).

A decrease in the hippocampal astrocytic density was observed in male tree shrews, which were exposed to social stress and these changes were correlated with the hippocampal volume reduction (Czeh *et al*, 2006). Also an in-house study demonstrates an impoverished hippocampal astroglial morphology and reduced hippocampal volume in adult Wistar rats that underwent early life deprivation (Opacka-Juffry et al., 2008).

When it comes to clinical evidence, a post mortem histological analysis of the frontal cortex showed a decrease in number of glia in major depressive disorder patients (Cotter *et al*, 2001a, 2002; Rajkowska *et al*, 1999) and reduced glial density and glia/neuron ratio has been observed in amygdala (Bowley *et al*, 2002; Hamidi *et al*, 2004). Hamidi *et al* (2004) also showed oligodendrocyte deterioration in the amygdala region of MDD patients. Study carried out by Cotter *et al* (2002) suggested a decrease in glial cell density in the prefrontal cortex region of major depressive disorder patient. Immunohistochemical studies using glial fibrillary acidic protein (GFAP), a selective marker of astroglia, also showed a decrease in GFAP stained cell count in prefrontal cortex and hippocampal CA1 and CA2 areas in MDD patients and interestingly no parallel changes were observed in hippocampal neurons (Miguel-Hidalgo *et al*, 2000;

Muller *et al*, 2001). These and other observations have led to the hypothesis that glial cell dysfunction may contribute to the pathogenesis of depressive disorders (Cotter *et al*, 2001).

The present study will assess tissue remodeling and astroglial changes in the brain regions implicated in the neurobiology / neuropathology of depression and effects of early life stress. The regions of interest are hippocampus, amygdala (associated with mood and memory of recent events), prefrontal cortex, raphe nucleus (associated with serotonin receptors) and nucleus accumbens (responsible for perception of pleasure and reward) (Bannerman *et al*, 2004; McEwen, 2000; Fuchs and Flugge, 1998).

1.8 Glia - Neurone interaction

Over the past decade, there is increasing evidence that neurones and glia cell interaction plays a role in the plastic morphological and functional relationship (Pfrieger and Barres, 1996). Glia-neurone interactions control various important processes of brain development like neurogenesis (Lim & Alvarez-Buylla 1999), myelination (Bhat, 2003), synapse formation (Slezak & Pfrieger, 2003; Ullian et al., 2004), neuronal migration (Nadarajah & Parnavelas, 2002), proliferation (Gomes et al., 1999), neuroplasticity (Azcoitia et al., 2010) and differentiation (Garcia-Abreu et al., 1995). Both glia and neurones secrete important soluble factors such as neurotransmitters, hormones and growth factors which have been implicated in the nervous system morphogenesis (Gomes, 2001).

Glia – Neurone interaction is critical for the neuronal survival, both in vitro and in vivo, as glia provides trophic and nutritive support (Meyer-Franke et al., 1995; Tsacopoulos and Magistretti, 1996). It is also crucial for the maintenance of homeostasis and central nervous system (Giaume et al., 2007; Rossi and Volterra, 2009). The glia - neurone cross talk includes signalling via ion fluxes, cell adhesion molecules and glutamate neurotransmitters (Marchetti, 1997; Fields and Graham, 2002)

Neurones control the glia-mediated activation of the two major neurotransmitter systems - acetylcholine and noradrenaline and deficiency of these neurotransmitter systems are linked to Parkinson's and Alzheimer's disease suggesting the pivotal role of the glia-neurone interaction (Camevale et al., 2007). Multiple sclerosis (MS), a disease caused by loss of myelin resulting into abnormal conduction of action potential, is considered to be due to disruption in the glia-neurone communication which is pivotal for the axonal conduction and myelination (Fields and Graham, 2002). Loss of axons and neurones is observed in patients suffering from MS (Bjartmar et al., 2001).

Alterations of the both glia and neurones has been observed in the both preclinical and clinical studies suggesting the importance of these two cell types and understanding the mechanisms involved in their bidirectional interaction could provide valuable insights in further understanding of the neurodegenerative disease which may further yield into valuable therapeutic strategy.

1.9 Serotonin and its receptors

Serotonin is known as important brain neurotransmitter which is involved in the regulation of human behavior, as well as in the pathophysiology of psychiatric disorders.

1.9.1 Structure, synthesis and metabolism of serotonin

Serotonin, chemically known as 5-hydroxytryptamine, is a biogenic monoamine found in almost all organisms. Tryptophan, an essential amino acid which we get through diet, is a precursor to serotonin. In the neuron, tryptophan is converted into 5-hydroxytryptophan (5-HTP) via action of enzyme tryptophan hydroxylase (TPH). 5-HTP in turn is converted into serotonin by aromatic amino acid decarboxylase (AADC). Serotonin is then transported into vesicles by the vesicular monoamine transporter (VMAT) and stored in the neural endings before being released into the synaptic cleft. The serotonin transporter (5-HTT) clears serotonin from the synaptic cleft by transporting it back into the nerve terminal. Further, serotonin metabolises into 5-hydroxyindole acetic acid (5-HIAA) by monoamine oxidase (MAO) enzyme activity.

1.9.2 Types of serotonergic receptors

Serotonin receptors, except one, are guanine nucleotide – binding protein (G-protein) coupled receptors. The released serotonin effects are mediated by a large number of receptors. So far 15 subtypes of serotonin receptors have been characterised (Bockaert et al, 2006). The serotonin receptor subtypes are divided into seven classes i.e. 5HT1 – 5HT7 depending on ligand affinity, molecular structure and intracellular transduction mechanism. 5-HT3 receptors are the only subtype which is ligand-gated ion channels

which possess different mode of action compared to others (Barnes and Sharp, 1999). The 5-HT1A (also located in somatodendritic and postsynaptic site), 5HT-1B and 5HT-1D (located in nerve terminals) receptors act as autoreceptors and exerts inhibitory effects on the serotonergic transmission (Hoyer et al, 2002). 5HT4 receptor has been linked with increased motor activity in animals exposed to stress and is observed to be altered in animal models of depression (Rymar et al, 2007).

1.9.3 Role of serotonin and its effects in the brain

Various physiological and behavioral processes mainly cognition (learning and memory), anxiety, feeding, sleep, pain and aggression are mediated through the serotonergic system (Whitaker-Azmitia et al., 1996; Lucki, 1998; Hoyer et al., 2002; David et al., 2005). The monoamine 5HT is know to be involved in the pathophysiology and therapy of depression (Stockmeier, 2003). Alteration in the 5HT neurotransmission has been implicated in various neurological disorders such as autism, depression (Lucki, 1998), Huntington's disease (Yohrling IV et al., 2002), Alzheimer's disease (Schmitt et al., 2006), schizophrenia and attention deficit disorder (Azmitia, 1999). Studies have shown altered level of 5HT and receptor densities in animal models of early life deprivation, stress and anxiety (Drevets et al., 2000, Hoyer et al., 2002) and also in patients suffering from depression (Arango et al., 1995). Studies have also confirmed altered serotonergic function in the hippocampus of rats underwent social isolation (Marsden et al., 2011) in response to stress using both footshock and conditioned emotional response (Fulford and Marsden, 1998a; Muchimapura et al., 2002). Furthermore, serotonin system has been correlated with addictive personality disorders

such as gambling and substance abuse (Campbell-Meiklejohn et al., 2011). An experimental study in rats has shown long term learning and memory deficits in response to early developmental exposure to drugs of abuse (methamphetamine), which are known to disrupt the 5HT system (Williams et al. 2002; 2003; review by Parrott and Marsden, 2006).

However, among the different 5HT receptor subtypes, the 5-HT1A receptors are considered to be the most influential (David et al., 2005) and have gained more attention in regards to 5HT developmental effects (Patel and Zhou 2005). The important regulatory role of the 5HT1A receptors is to mediate the negative feedback and these are known to desensitize due to the antidepressant treatment (David et al., 2005). In rodents, 5-HT1A binding sites have been identified during the neonatal development stage mostly in the neurons and glia in the hippocampus and cortex along with brain stem (Daval *et al.* 1987; Patel and Zhou 2005).

Increased 5-HT1A receptor binding in the prefrontal cortex of nonviolent suicides compared to violent suicides and controls has been reported (Arrango et al., 1995). The 5-HT1A autoreceptor function is reduced in rats exposed to high glucocorticoid dose or under chronic stress (Fairchild et al., 2003). In another study conducted by Joels and colleagues have reported increased 5HT1A receptor sensitivity in the CA1 pyramidal cells of hippocampus due to stress acting via glucocorticoid receptors (Hesen and Joels, 1996). However, in contrast to this finding, adrenalectomy in rats has shown to increase 5-HT1A binding in cortex (Kuroda et al., 1994). Rats implanted with slow release corticosterone pellet (for 7 days) has shown to induce long-term alterations in the

cortical 5-HT1A receptor binding along with persistent decrease in whole body and adrenal weight (Bush et al., 2003).

1.10 Oxytocin and its receptors

Oxytocin (OT) is a central nervous system (CNS) neuropeptide consisting of nine amino acids. OT is also known as a nonapeptide and was discovered by Sir Henry Dale in the year 1906. The name oxytocin was derived from the greek words – ωκνξ and τοκοχξ meaning "quick birth" (Dale, 1906). In 1953, OT was the first peptide hormone to be sequenced and synthesised by Vincent du Vigneaud, for this work he was awarded the Nobel Prize in 1955 (du Vigneaud, 1956).

OT is mainly produced and synthesised in magnocellular neurons of the hypothalamic paraventricular (PVN) and supraoptic nuclei. OT is secreted into the periphery *via* the posterior pituitary where it mediates effects on uterine contractions, milk ejection in lactation and cardiovascular control, among others. The central actions of the OT are mediated via oxytocin receptors (OTR) which are distributed widely in the CNS. Till date only one OTR is identified which is a member of class 1 – G protein coupled receptor family (Gimply, 2001). OTR are located in various brain regions which include - the ventromedial nucleus of the hypothalamus, the amygdala, the lateral septum, the bed nucleus of the stria terminalis, the anterior olfactory nucleus, the preoptic and ventral tegmental areas, and the hippocampus (Li et al, 1997).

1.10.1 Role of oxytocin and its effects in the brain

Oxytocin was originally considered as a "maternal hormone" due to its functional role in the regulation of reproductive functions and maternal behaviour. However, recently OT has been implicated in a various other behaviour and neurochemical processes (Neumann, 2008).

Oxytocin plays important role in respect to social interaction and bonding which is essential for the positive health benefits and survival of species as it favours sexual behaviour, protection, environment and brain development. However, social isolation leads to various behavioural pathologies such as autism and depression characterised by neurochemical pathway deficits (Carter et al, 2008; Neumann, 2009).

OT is a hypophysiotrophic hormone which under physiological condition controls the secretion of the adreno-corticotropic hormone in stressful situation. This oxytocin-induced ACTH secretion is blocked by adrenal glucocorticoid hormone (Gibbs, 1986). Hence, oxytocin regulates the HPA axis functioning by reducing ACTH release which in contrast is helpful in treating stress-HPA induced pathologies (Boutet et al, 2006).

The oxytocinergic system is equally developed in both males and females; still it is under strong influence by the female steroid hormone – estrogen. Strong connections between estrogen and OT lead to sex differences (Yamaguchi et al, 1979; Schumacher et al, 1993). Estrogen hormone stimulates synthesis and release of OT and increases the OTR in brain regions. A recent study has shown involvement of estrogen β receptor mediated oxytocin release (Somponpun and Sladek, 2002) and estrogen α receptor

mediated OTR increase in amygdala (Choleris et al, 2003). Interestingly, oxytocin autoreceptors have been identified on some of the oxytocinergic neurons due to these autoreceptors OT is able to stimulate its own release (Moos et al, 1984; Freund-Mercier and Stoeckel, 1995).

OT administration may induce anxiolytic and sedative effects with high doses. These effects are mediated via amygdala which contains high OTR and plays important role in social recognition (Uvnas-Moberg et al, 1994; Ferguson et al, 2001).

The other effects of OT are increase in nociceptive thresholds (Lund et al, 2002), decrease in heart rate and blood pressure (Uvnäs-Moberg, 1998), influences secretion of insulin and glucagon (Dunning et al, 1984) and induce anti-oxidative effect (Moosmann and Behl, 2002).

1.11 Main aims and hypotheses of the thesis

This doctoral thesis consists of four studies. The first one (Chapter 3) was aimed to assess the long-term effects of the early life postnatal deprivation (ED) on the brain regional volume and astroglia morphology in two rat strains of different responsiveness to stress, Wistar and Lewis, normo- and hypo-responsive, respectively. Based on the previous findings, it was hypothesised that ED would lead to a long-term reduction in the volume of the hippocampus and impoverishment of astroglial morphology in Wistars with no changes in Lewis rats. The second study (Chapter 4) on rats exposed prenatally to glucocorticoids (dexamethasone) during E16-E19 hypothesised that *in utero* dexamethasone administration would lead to hippocampal volume loss and impairements in GFAP-positive astroglial morphology in adult offspring.

The third study (Chapter 5) aimed to investigate the long-term effects of prenatal dexamethasone administration on the oxytocin and 5-HT1A serotonin receptor binding in male and female rats. These receptors were selected for their involvement in the stress response, and because of the associations between prenatal stress, exposure to glucocorticoids and risk of neuropsychiatric disorders in adult life. The fact that very few studies have researched the effects of prenatal manipulations affecting glucocorticoid programming motivated the present approach.

In addition, in the fourth study (Chapter 6), the *in vitro* effects of dexamethasone on neural stem cells were investigated, in order to assess the effects of dexamethasone on the cell proliferation and differentiation, and on glial cell markers. This part of thesis referred to the mechanisms of stress/glucocorticoid-dependent hippocampal volume

loss; it has been known that neural stem cell activity continues in the hippocampus until adulthood and its impairment can theoretically contribute to volume losses of this brain region.

Chapter 2

Methodology

This chapter consists of the descriptions of all the methodologies used for the present dissertation.

2.1 Animal models

2.1.1 Early Deprivation

2.1.1.1 Experimental Design and Materials

The present study was designed as an experiment with between group comparisons. Two rat strains of different responsiveness to stress - Wistar and Lewis were compared, also treatment effect were compared between the groups: non-handled (NH), early handled (EH) and early deprived (ED). It is aimed to estimate the volume of the specific brain regions implicated in stress and depression. The brain regions considered for analysis were: dorsal hippocampus, basolateral amygdala and nucleus accumbens (see Figure 2.6).

The material, perfused and frozen male rat brains, was offered by Dr Christopher Pryce of the Swiss Federal Institute of Technology (ETH) in Zurich. It included 32 brains, six per group: non-handled control, early handled and early maternally deprived (ED), representing Wistar strain and 3 non-handled, 5 early handled and 6 early deprived representing Lewis strain (exception being Lewis NH where n=1 was used for total brain volume analysis). The choice of rat strains was informed by the previous research: the Wistar strain has been found to respond to ED - behavioural and receptor changes have been observed (Leventopoulos et al., 2009). The Lewis strain seems to show no negative behavioural response to ED (Personal communication – Dr Pryce).

The number of animals (n=6 per group) is based on the previous collaborative studies carried out at the Roehampton University and the Swiss Federal Institute of Technology (ETH) in Zurich (Leventopoulos et al., 2007 and 2009; Opacka-Juffry et al., 2008) and reflects the ethical restrictions that recommend reducing the numbers

of animals to the necessary minimum. The behavioural assessment was performed at the ETH in Zurich (Pryce, personal communication) and post-mortem analyses were carried out at the Clinical Laboratory, Department of Life Sciences (previously School of Human and Life Sciences) University of Roehampton.

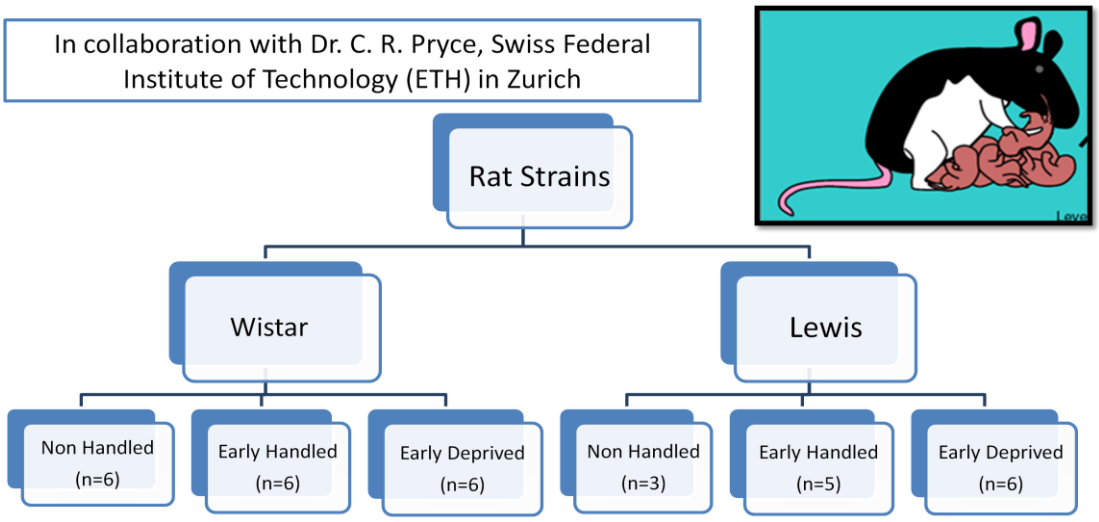

Figure 2.1 - Graphical representation of the early deprivation model experimental design employed for brain regional volume and astroglia morphology analysis (as used in chapter 3).

2.1.1.2 Animals and their treatment

Wistar rats (Harlan, NL) were bred and maintained at the animal facility of the Swiss Federal Institute of Technology, Schwerzenbach, Switzerland on a reversed light–dark cycle (off 07:00–19:00 h) at 21^0 C and 50–60% humidity, with water and food available ad libitum. All experiments were carried out in agreement with Swiss Cantonal Veterinary Office regulations for animal experimentation. On postnatal day 1 (PND) each of ten early deprivation (ED) litters, ten early handled (EH) litters and ten non-handled (NH, control) litters were culled to four male and four female pups.

ED pups were isolated from dam and littermates 4 h daily (12:00–16:00 h) on PND 1–14 at room temperature (21^0 C) during the dark phase (according to Ruedi-Bettschen et al., 2005). EH pups were removed from the dam for 1 minute in order to control for the human handling component of ED. NH dams and litters were kept undisturbed and the colony room was entered for provision of food and water and observation of maternal behaviour only. The ED procedure consisted of removal of the dam from the home cage, removal of the pups and individual placement on sawdust in a PVC separation apparatus comprising single pup compartments of 10 x 10 x 20 (H) cm, and return of the dam to the home cage during ED. At the end of ED, the dam was again removed from the home cage, the pups were returned to it, and then dam returned to home cage with pups. The EH procedure consisted of removal of the dam from the home cage, removal of the litter to a different cage and then immediate return to the home cage, and return of the dam to home cage with pups. Subjects were weaned at PND21 and same-sex littermates were caged together. Following weaning, all subjects were left to develop undisturbed except for regular cage-cleaning once a week until the start of behavioural testing during adulthood (4–6 months). Subjects were sacrificed at the age of 12 months. Rats were decapitated and brains were frozen at -50^0 C in isopentane/dry ice/ethanol and stored at -80^0 C. Frozen brains were transferred on dry ice from Zurich to London for processing.

2.1.2 Prenatal Dexamethasone

2.1.2.1 Experimental Design and Materials

The present study was designed as an experiment to investigate (a) effects of prenatal dexamethasone treatment on the volume of the brain regions, dorsal hippocampus, basolateral amygdala and nucleus accumbens and morphology of astroglial cells, and

(b) effects of prenatal dexamethasone (DEX) treatment on the regulatory brain receptor systems (Figure 2.2).

The material for the volumetric study (a) consisted of perfused fixed male Sprague-Dawley rat brains, while that for the receptor study (b) consisted of fresh-frozen male and female Sprague-Dawley rat brains, offered by Professor Glenda Gilles of the Faculty of Medicine, Imperial College in London. It includes 12 brains each for study (a) and (b), six per group: control and DEX treated. The number of animals (n=6 per group) reflects the ethical restrictions that recommend reducing the numbers of animals to the necessary minimum. The post-mortem analysis was carried out at the Clinical Laboratory, Department of Life Sciences, University of Roehampton.

2.1.2.2 Animals

Sprague–Dawley (SD) rats (Harlan Olac, Blackthorn, Beicester, Oxfordshire, UK) were housed and timed matings were carried out in the Comparative Biology Unit at Charing Cross Hospital (Faculty of Medicine, Imperial College, London). All animal procedures were carried out under the license in accordance with the United Kingdom Animals Scientific Procedures Act of 1986. SD rats were under controlled lighting (on 0800–2000 h), temperature (21–23^{o}C) and humidity (63%), with standard rat chow and drinking water (except as described below) provided *ad libitum*. Rats both, males and females were caged separately, and allowed to acclimatise to their new environment for 1 week, after which groups of one male and two female rats were housed together overnight and the presence of vaginal plugs the following morning was taken to confirm mating; pregnancy was confirmed approximately 6 days later by palpation. The timed pregnant rats were housed five per cage until gestational day (GD) 15, when they were caged singly in preparation

for giving birth. From GD 19/20 pregnant rats were monitored several times a day and the day of birth was defined as day 0. Offspring were weaned at 3 weeks, after which male and female animals were housed separately in standard, wire-topped cages in groups of five per cage and were allowed to grow to young adulthood with no further treatment. At 68±2 days of age animals were killed by decapitation between 0900 and 1000 h to minimize effects associated with circadian rhythms.

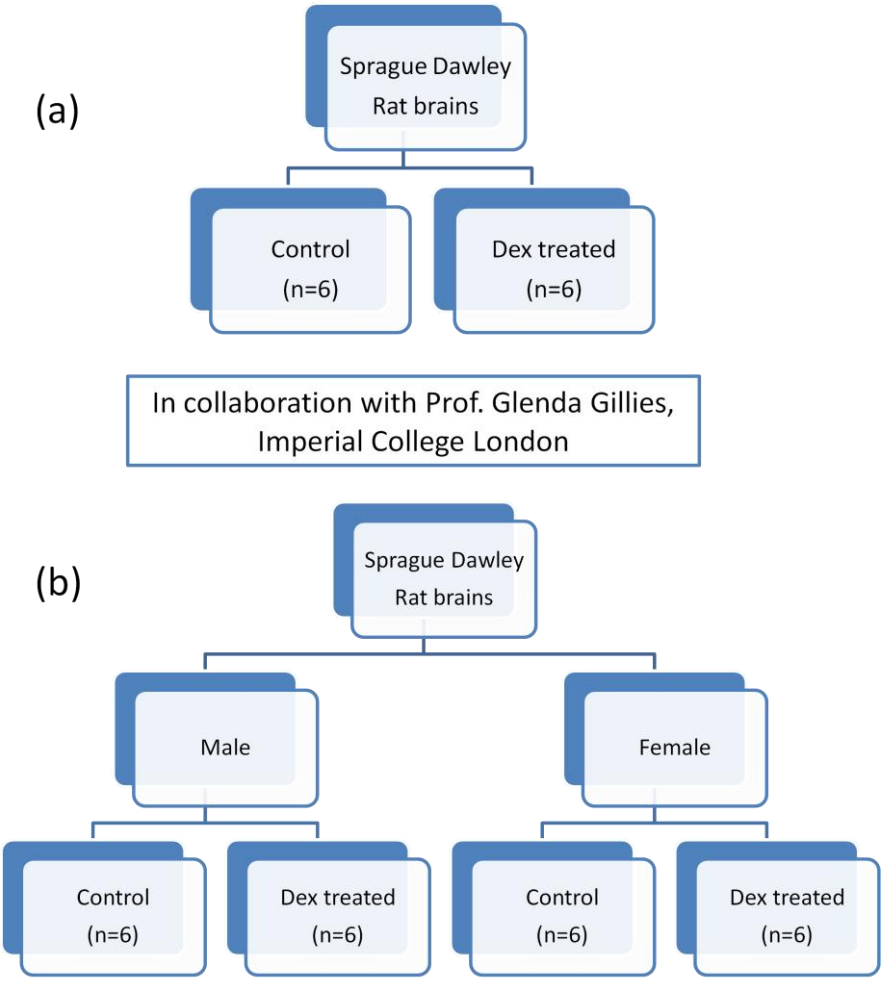

Figure 2.2 - Graphical representation of the prenatal Dex treatment experimental design (a) perfused fixed male rat brains for volumetric and morphological analysis (as used in chapter 4) and (b) fresh frozen male and female rat brains for receptor analysis (as used in chapter 5)

2.1.2.3 Dexamethasone Treatment

Dexamethasone (Faulding Pharmaceuticals Plc., Royal Leamington Spa, UK) was administered non-invasively using the method established by McArthur et al (2005) and is done by adding dexamethasone to the drinking water of pregnant or nursing dams between embryonic days 16–19 (E16–E19, 0.5 µg/ml) or postnatal days 1–7 (P1–P7, 1 µg/ml). This route of administration also avoids potential confounding effects of injection stress and also handling of the newborn pups, which may itself produce long-lasting effects on behaviour and brain function in later life (Meaney et al, 2002). Using this oral route, dexamethasone is able to reach the developing foetuses or neonates via the placenta (Funkhouser et al, 1978) or milk (Tainturier et al, 1982), respectively.

Previous studies carried out by McArthur et al (2005) used available pharmacokinetic data that allows estimating, from the amount of dexamethasone ingested by the dams, that similar concentrations are achieved in the fetus and neonate (~30 ng/ml). Allowing for the greater GC potency of dexamethasone, which is reported to be between one and two orders of magnitude greater than that of corticosterone, it would appear that the levels attained can be considered comparable to the GC potency that prevails following stress induced activation of the maternal HPA axis. On the basis that the mothers' daily intake of water was approximately 50 ml (McArthur et al, 2005), allows approximate calculation of the dexamethasone dosage (approximately 75 and 150 µg/kg/day for prenatal and neonatal treatments, respectively) which is within the range used clinically to mature the fetal lung in cases of threatened premature birth. As previously reported (McArthur et al, 2005; Theogaraj et al, 2005; McArthur et al, 2006a), these dexamethasone treatments had

no significant effects on the outcome of the pregnancies, no discernable influence on maternal behavior and no effect on adult body weight.

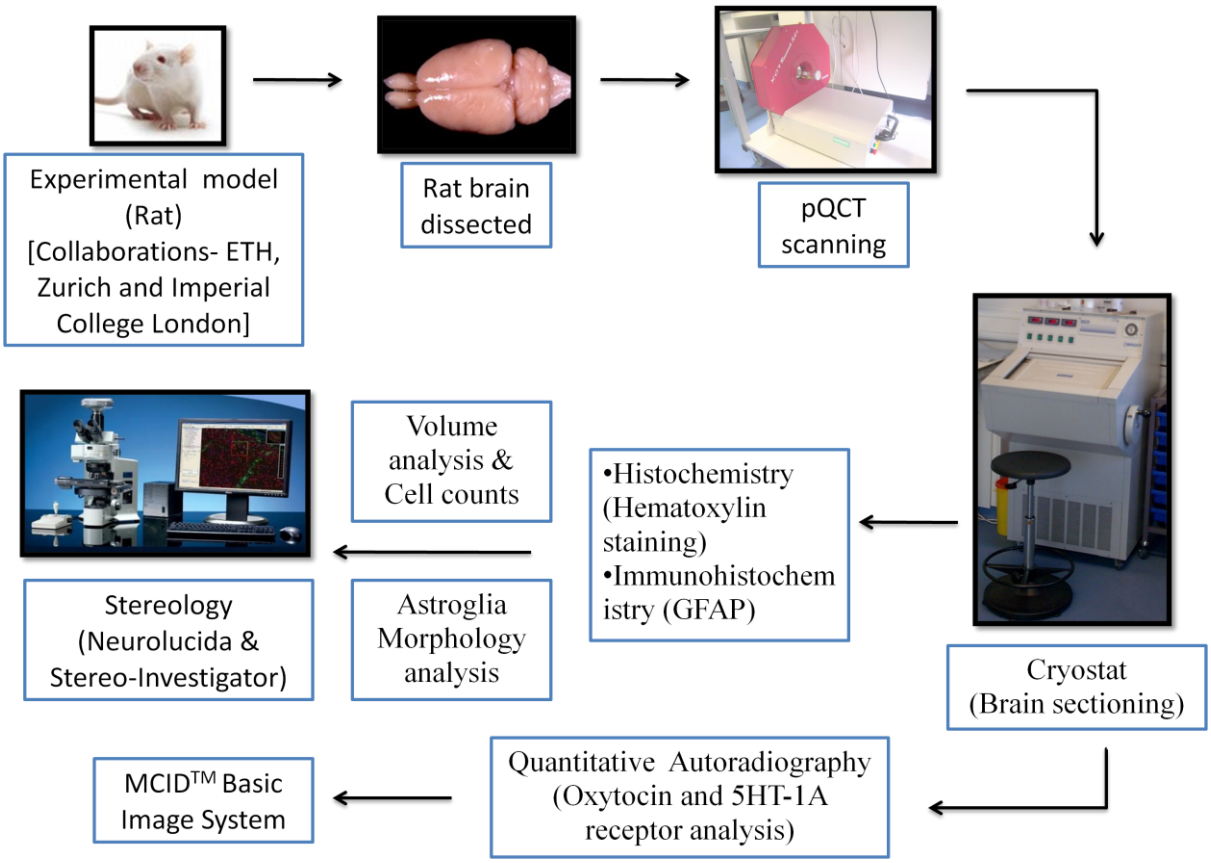

Figure 2.3 - Diagrammatic representation of the overall methodology used in the present study.

The above flowchart indicates stepwise experimental methods and techniques used in the thesis (as used in chapter 3, 4 and 5). Experimental rat models were sacrificed, dissected and were scanned to measure total brain volume. Further cryosectioned and stained for volume, cell count, morphology and autoradiography analysis. However, methods employed in Chapter 6 were not shown in this diagram.

2.2 Brain tissue preparation

The rats were deeply anesthetised with an overdose of sodium pentobarbital and intracardially perfused with phosphate-buffered saline (PBS) and then with 4% formaldehyde pH 7.4. Brains were immediately removed, post fixed overnight in the same fixative, and cryoprotected in glycerol/glycol solution. Those procedures were carried out at the ETH, Zurich (ED studies) or Imperial College London (DEX study). In DEX study (b), rat brains were quickly removed and frozen at -40^0C to achieve best results with the receptor autoradiography. The brains were frozen and transferred on dry ice to Roehampton University and kept at −80 °C until pQCT total volume analysis and cryostat sectioning.

2.3 Brain imaging - Peripheral Quantitative Computed Tomography (pQCT)

The pQCT (Stratec Research SA+ scanner, Stratec Medizintechnik, Pforzheim, Germany) is an X-ray scanner based on the translation-rotation principle - where the X-ray beam passes through the specimen, its intensity is attenuated. Due to high atomic number compared to air, the high density is associated with a high attenuation of the beam which results into scan. pQCT has a relatively small gantry (compared to the usual CT scan) allowing the assessments of small objects (< 8 cm in diameter). The major difference with clinical CT scans is that it has a smaller gantry (8 cm vs 80 cm) and relatively higher resolution (70μ vs 200μ). The major difference with μCT (micro CT) is that is has a larger gantry (for μCT typically ~ 1cm) a lower resolution (70μ vs 20μ).

Most of all, the pQCT produces images (slice or tomography) where, for each pixel, the density is quantitatively measured. The pQCT has been widely used in the clinical field for investigation of the shape and density of the distal radius (Boonen et al. 1997). Some models have been adapted for the *in vivo* investigation of small animals (Turner et al. 2001 or morphology. On the other hand, such tools have been used for the *in vitro* investigation of human cancellous bone density (Bailey et al. 1999).

pQCT possesses 12 semiconductor detectors with amplifiers and a microfocus tube with 50-µm spot size (voltage 45–60 kV; anode current = 0.2– 0.5 mA; mean X-ray energy 38–45 keV; width of energy beam after filtering = 25 keV). It provides a central gantry opening of 50 mm and uses attenuation profiles obtained at 1° angles to compute cross-sectional images. All images were obtained at a section thickness of 500 µm and at an in-plane pixel size of 70 × 70 µm.

2.3.1 Key features of pQCT

pQCT serves as a cost-effective means for detecting and characterizing soft-tissue structures and skeletal abnormalities. It requires less sample preparation time and relatively simple data post-processing which makes it user friendly.

pQCT was not a useful tool since its introduction for laboratory investigations of soft tissues and small rodents, mainly because of limitations in spatial resolution. However, recent technical advances have revolutionised the clinical diagnostic practice and have made it practical to obtain high-resolution CT images of small-specimens mainly soft tissue and animals during research investigations.

pQCT has become a mature technique that can augment or replace histological analysis in many research applications, with a major advantage being that it does not interfere with any additional anatomical or mechanical study on fresh or fixed tissue. The major application of pQCT to date has been in quantifying the density and architecture of bone, but there are rapidly growing applications in vascular studies and in the characterization of the phenotype of transgenic and knockout animal models during preclinical investigations. pQCT will probably become a standard tool in many laboratories in the future, as it provides accurate and precise data.

2.3.2 pQCT Method

Rat brains stored at -800C were scanned via the peripheral quantitative computed tomography technique (pQCT) on a Stratec Research SA+ scanner (Stratec Medizintechnik, Pforzheim, Germany) with settings of 50.5 V, 261.1 mA and 13.2 W. Serial Coronal CT scans (302 x 302) with a pixel size of 0.1mm were performed at 1mm intervals covering the entire brain region. This technique is normally used for hard tissue (bone) analysis and the present application was novel and required method development as described below.

Data analysis to measure volume was carried out by using software Avizo (version 5; Mercury Computer Systems, Chelmsford, MA, USA) (Options: full brain cross-section selected with the 'magic wand' tool in 'Label Field', volumes obtained with the 'Surface Gen' option).

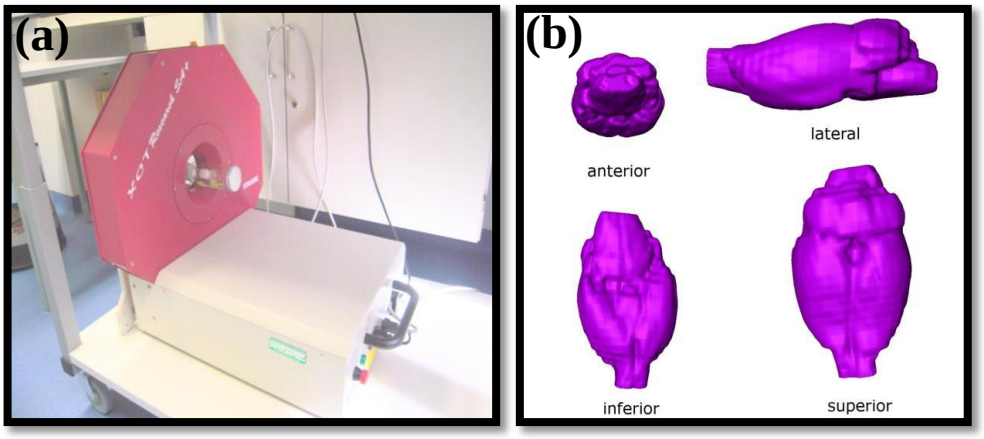

Figure 2.4 - (a) Peripheral quantitative computed tomography (pQCT) Stratec Research SA+ scanner (Stratec Medizintechnik, Pforzheim, Germany) and (b) images obtained from it.

2.3.3 Brain Holder device

A bespoke brain holder device (Figure 2.5) was developed to maintain freezing conditions for rat brains. It consisted of several parts:

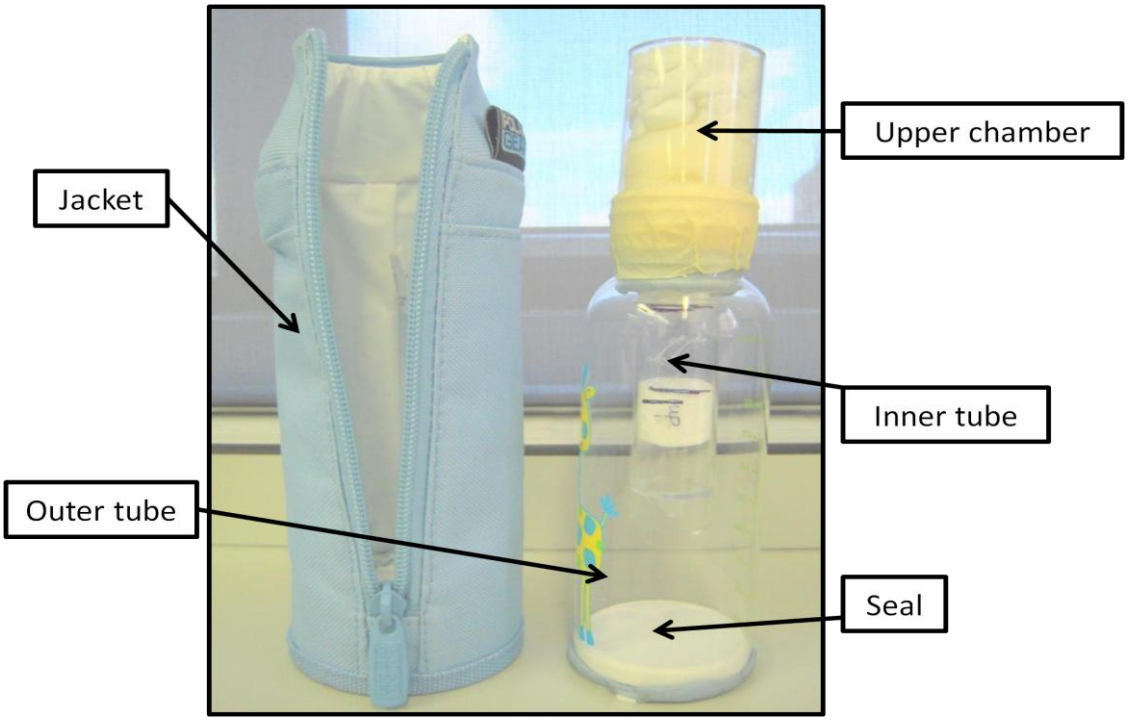

Figure 2.5 - Image of the bespoke brain holder device designed for the present study.

- *Upper chamber*, which went into the pQCT holder, filled with sponge and thermacol to avoid water dripping into pQCT.
- *Inner tube*, which held brain tissue and acts as an interface between brain & dry ice.
- *Seal*, which held dry ice.
- *Outer tube*, which contained dry ice to provide freezing temperature.
- *Jacket*, which reduced condensation.

2.4. Cryostat sectioning

After initially removing the olfactory bulb, both perfused fixed and frozen rat brain tissue was supported in optimum cutting tissue (OCT) embedding medium (RALamb Ltd, Eastbourne, UK) and equilibrated in the cryostat (Bright Instruments Co. Ltd, UK) in preparation for cutting coronal sections in an anterior – posterior direction. A stainless steel knife was used at an angle of 15 degrees. The specimen temperature was set at -15^0C with the chamber temperature at -21^0C. Coronal sections were cut at 25µm thickness for volumetry and morphology analysis and 20µm thickness for receptor work, according to the rat brain atlas of Paxinos and Watson (5^{th} Edition) and collected on polylysine glass slides (3 sections per slide). The sectioning procedure was designed such that the following brain regions of interest (ROIs) were represented (in brackets A/P coordinates from bregma, in mm): nucleus accumbens core (3.00 to 2.16), basolateral amygdala (-1.72 to -2.28), dorsal hippocampus (−2.04 to -4.68). Figure 2.6 and Table 2.1 below shows detailed information.

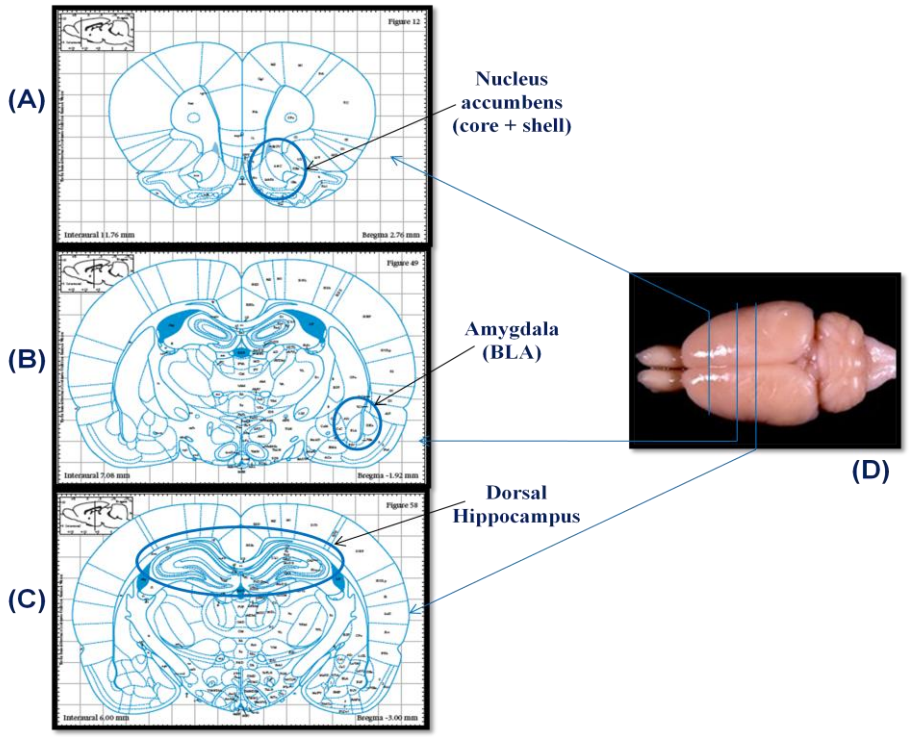

Figure 2.6 - Diagrammatic representation of coronal rat brain sections showing the specified region of interest in rat atlas. (Paxinos & Watson – Rat brain atlas, 5th Edition)

A. *Nucleus accumbens region at bregma – 2.76mm, figure 12 of the Atlas*
B. *Basolateral amygdala region at bregma - 1.92mm, figure 49 of the Atlas*
C. *Dorsal hippocampus region at bregma - -3.00mm, figure 58 of the Atlas*

Brain region	Location	
	Starting Bregma	**Figure no**
Prelimbic cortex (PRL)	2.76	12
Accumbens nucleus, core (AcbC)	1.80	18
Lateral septal nucleus, dorsal part (LSD)	1.08	24
Basolateral amygdaloid nucleus, anterior part (BLA)	-1.92	49
Paraventricular hypothalamic nucleus, medial magnocellular part (PaMM)	-1.92	49
Ventromedial hypothalamic nucleus (VMH)	-1.92	49
Central amygdaloid nucleus (CeM)	-1.92	49
Dorsal hippocampus (CA1, CA2, CA3, DG)	-3.24	60
Ventral hippocampus (CA1, CA2, CA3, DG)	-4.92	74
Dorsal and ventral raphe nucleus (DR & VR)	-7.32	94
Subiculum, transition area (SubTr)	-7.32	94

Table 2.1 - Brain regions analysed for oxytocin and 5HT-1A receptors using autoradiography and its representative location in the rat brain atlas (Paxinos & Watson – Rat brain atlas, 5th Edition).

2.5 Histological procedures

2.5.1 Hematoxylin staining

The frozen rat brain sections (-80^{0}C) mounted on the polysine slides (Menzel-Glaser^R, UK) (3 sections per slide) were taken out from the freezer and allowed to air dry for 1 hour on tray at room temperature. Sections were then rehydrated in phosphate buffered saline (PBS, Oxoid, UK) (pH 7.4) (dissolve 10 PBS tablets in 1000 ml of distilled water) for 5 minutes at room temperature. Rehydration was carried out in order to prevent the tissue damage due to swift changes in temperatures and ensure good cell membrane permeability. Sections were then stained for cell nuclei using hematoxylin (Sigma-Aldrich, UK) for 2 minutes at room temperature. They were rinsed in distilled water by three dippings in order to remove excess stain. In order to reduce the background staining differentiation was done using 1% HCl – 70% alcohol by 3 dippings. Again rinsing was done using distilled water by several dippings and then with PBS for 5 minutes at room temperature (RT). Sections were then washed using distilled water for 5 minutes at room temperature. The sections were then dehydrated using ethanol at increasing concentrations (50%, 70%, 90% and 100%) for 5 minutes each at room temperature. Sections were then air dried for 15 minutes and protected with histological mounting medium (National diagnostics, UK) and covered with a cover slip (Fisher brand, UK).

2.6 Immunohistochemistry procedures

2.6.1 GFAP staining

Day 1:

- The frozen rat brain sections (stored at -80^{0}C) mounted on the polysine slides (3 sections per slide) were taken out from the freezer and allowed to air dry for 1 hour on tray at room temperature. The sections were encircled with a wax pen, which ensures uniform contact of section with the solution.
- Sections were then rehydrated in PBS (pH 7.4) for 15 minutes at room temperature. Rehydration is carried out to ensure good cell membrane permeability.
- The presence of peroxidase enzyme in the tissue causes high background staining. Hence peroxide activity in the brain sections was inactivated by treating them with a peroxide inactivating solution containing 1.5% hydrogen peroxide (Sigma-Aldrich, UK) for 15 minutes.
- This was followed by rinsing with PBS for 15 minutes at room temperature to remove excess solution.
- Sections were then incubated in the blocking solution (1ml of normal horse serum + 8ml of 0.1% PBST + 1ml of 0.5% sodium azide solution) for 1 hour at room temperature on a tray. Extreme care was taken to prevent tissue drying. The purpose of blocking was to prevent non-specific antibody binding.
- Again, the sections were rinsed with PBS – 5 minutes, PBST – 10 minutes, PBS – 5 minutes, at room temperature remove the excess blocking solution.

- Sections were then incubated with a primary anti-GFAP monoclonal antibody (Sigma-Aldrich, UK) solution at 1:500 concentration and then placed in the fridge at 4^0c for 24 hours incubation.

Day 2:

- On the following day, the sections were washed with PBS for 15 minutes at room temperature, to remove any unbound antibody.
- Sections were then incubated with ready to use anti – mouse IgG biotinylated antibody – secondary antibody (Vectastain Elite ABC kit, Vector Laboratories) for 30 minutes at room temperature. The addition of normal horse serum (NHS) prevents non- specific antibody binding and prevents high background staining.
- Towards the end of secondary antibody incubation the Avidin-Biotinylated horseradish peroxidise macromolecular complex (ABC solution, Vectastain Elite ABC kit, Vector Laboratories) was prepared and allowed to stand for 30 minutes at room temperature. This earlier preparation and the standing time ensures optimal complex formation.
- Meanwhile, the sections were rinsed with PBS for 15 minutes to remove excess and unbound secondary antibody at room temperature.
- After 30 minutes, the sections were incubated in ABC solution for 20 minutes at room temperature.
- Before starting the rinse following the ABC incubation, the pre–made diaminobenzidine (DAB, Sigma-Aldrich, UK) solution was taken out from the freezer and allowed to thaw in dark.

- Meanwhile, the sections were rinsed in PBS for 15 minutes at room temperature.
- Sections were incubated in DAB solution for 5minutes.
- Rinsing in distilled water for 5x3 minutes using fresh distilled water every time. Care was taken to prevent spilling of DAB (potential teratogenic agent) and spills were inactivated with a concentrated bleach solution.
- The sections were air dried for 30 minutes, before dehydration in ethanol at increasing concentrations (50%, 70%, 90% and 100%) for 5 minutes each at room temperature.
- The sections were air dried and protected with histological mounting medium and covered with a cover slip. Then sections were visualized under the microscope fitted with Olympus BX51 camera and analysed using Neurolucida and StereoInvestigator, MBF packages.

2.7 Stereological procedures

2.7.1 Neuroanatomy analysis

In order to outline the anatomy of different brain regions a Paxinos and Watson (5^{th} edition) stereotaxic rat atlas was used. The rat atlas was also used as a reference during cryosectioning. Cross referencing the stained sections using rat atlas provides a clear boundary for different brain regions.

Dorsal hippocampus volume: Both left and right dorsal hippocampus was measured covering entire region CA1, CA2, CA3, DG and hilus.

Basolateral amygdala volume: Right side of the BLA was measured covering entire BLA. In some sections lateral amygdaloid nucleus, dorsolateral part was seen but it was demarked and only BLA was measured.

Nucleus accumbens volume: Right side of the nucleus accumbens was measured including both core and shell.

2.7.2 Sampling for volume estimation

Dorsal hippocampus

Twelve sections per brain were used with section interval of 10 covering bregma -2.04 mm to -4.68 mm of stereotaxic rat atlas.

Series 1	Series 2	Series 3	Series 4	Series 5	Series 6	Series 7	Series 8	Series 9	Series 10
1	2	3	4	5	6	7	8	9	10
11	12	13	14	15	16	17	18	19	20
21	22	23	24	25	26	27	28	29	30
31	32	33	34	35	36	37	38	39	40
41	42	43	44	45	46	47	48	49	50
51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70
71	72	73	74	75	76	77	78	79	80
81	82	83	84	85	86	87	88	89	90
91	92	93	94	95	96	97	98	99	100
101	102	103	104	105	106	107	108	109	110
111	112	113	114	115	116	117	118	119	120

Figure 2.7 Representation of the serial sectioning and sampling of a sample set with a section interval of 10.

Basolateral amygdala

Six per brain were used with section interval of 5 covering bregma -1.72 mm to -2.28 mm of the stereotaxic rat atlas.

Nucleus accumbens

Five per brain were used with section interval of 10 covering bregma 3 mm to 2.16 mm of the stereotaxic rat atlas.

2.7.3 Sampling for cell count estimation

Eight sections per brain were used with section interval of 10 covering bregma -2.04 mm to -4.68 mm of the stereotaxic rat atlas.

2.7.4 Volumetric analysis using Cavalieri principle – StereoInvestigator

Examination of the hematoxylin (HX) stained sections was performed by means of an Olympus BX51 microscope fitted with a motorized stage and video camera. Quantification of the HX stained volumes of dorsal hippocampus, basolateral amygdala and nucleus accumbens was done using Cavalieri principle (StereoInvestigator, Version 9, MicroBrightField, Inc., Germany). Starting at a random position, every tenth section was analyzed, leading to an average of 12 sections (for dorsal hippocampus), 6 sections (for basolateral amygdala) and 5 sections (nucleus accumbens) per subject. The same sets of consecutive sections were used for the estimation of volumes for both control and test groups. The Cavalieri principle was used to estimate the volumes using the stereology workstation and investigator was blind to groups. A point counting grid (PCG) (i.e. d=50 μm) was used for volume estimation to obtain maximum efficiency. Representative area per point (a/p) for the dorsal hippocampus was 2500μm^2 respectively. After applying the PCG on the sampled sections in a systematic-random manner, the number of points hitting the region of interest was counted. These point counts were used for the estimation of volumes using the following formula;

$$\text{Volume} = (a/p) \times \sum P \times \text{ssf} \times t,$$

where a/p represents the area of each point on the point counting grid; ΣP is the total number of points hitting the pyramidal layer or hemisphere; t is the mean section

thickness; and ssf (1/12) is the section sampling fraction (Gundersen, 1986; West et al., 1991).

The efficiency of sampling and volume estimation was checked by estimation of the coefficient of error (CE) and coefficient of variation (CV) (Gundersen and Jensen, 1987; West et al., 1991).

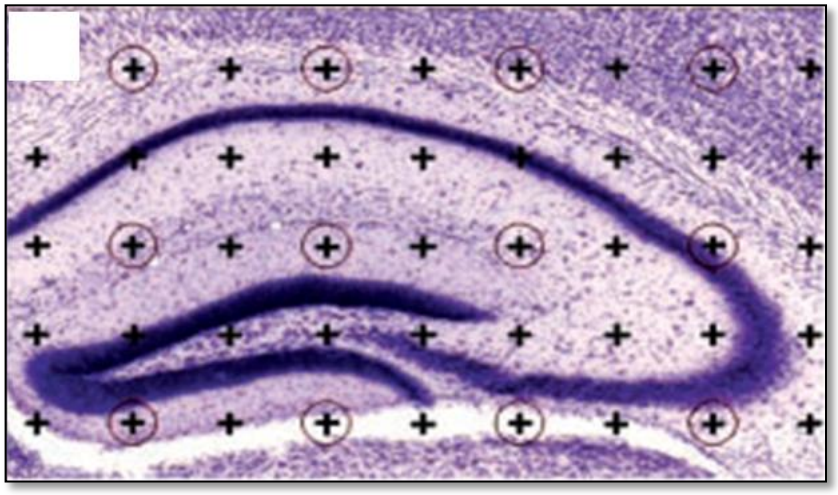

Figure 2.8 - Image showing in process measurement of hippocampus volume using cavalieri principle – Stereo-investigator.

The above figure shows grids (+) overlaying the region of interest. Using the stereoinvestigator tool grids intersecting inside the area of interest were selected. Once all the intersections on each section of same brain were marked, then the stereoinvestigator generates the volume of the measured region of interest.

2.7.5 Cell count analysis using Optical Fractionator – StereoInvestigator

Examination of the hematoxylin (HX) stained cells was performed by means of an Olympus BX51 microscope fitted with a video camera. Dorsal hippocampus subregional cell count was done for the areas CA1, CA2, CA3 and DG by using

optical fractionator (StereoInvestigator, Version 9, MicroBrightField, Inc., Germany).

An unbiased estimation of the total number of cells was obtained by choosing every 8th section according to the systematic random sampling procedure (West et al., 1991). A sampling area of 160/2500 μm^2 was found to be optimal for this study. Dissector height was 6µm and a 2µm guard zone at the top and bottom part of the section was excluded from the analysis at every step. Thus, a thickness sampling fraction of 6µm/t was used, where t represents the mean section thickness. A semi-automated stereology workstation for stereological analyses composed of a CCD digital camera (Nicon Coolpix E 4500, Tokyo, Japan), image capture card (Flash Point 3D, Integral Technologies, Indianapolis, IN, USA), personal computer and computer-controlled motorized specimen stage (Prior, Rockland, MA, USA), a microcator (Heidenhein, Traunreut, Germany) and a light microscope (Nicon, Eclips E 600, Tokyo, Japan) were used. HX-stained cells in the sub-regions of dorsal hippocampus were counted using a 100× Nicon Plan Apo objective (NA=1.30) which allowed accurate recognition. Each cell was counted according to the unbiased counting rules (West et al., 1991). Total cell count in sub-regions of dorsal hippocampus were estimated according to the formula given below:

$$N = \sum Q \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{tsf}$$

where ΣQ represents the total number of neurons counted in all optically sampled fields of the hippocampus; 'ssf' is section sampling fraction (1/8); 'asf' is area sampling fraction (160/2500); and 'tsf' is thickness sampling fraction (defined by

dissector height (6µm) divided by estimated mean section thickness) (West et al., 1991).

The efficiency of convenient number of sampled cells and parameters was checked by estimation of the coefficient of error (CE) and coefficient of variation (CV) (West et al., 1991).

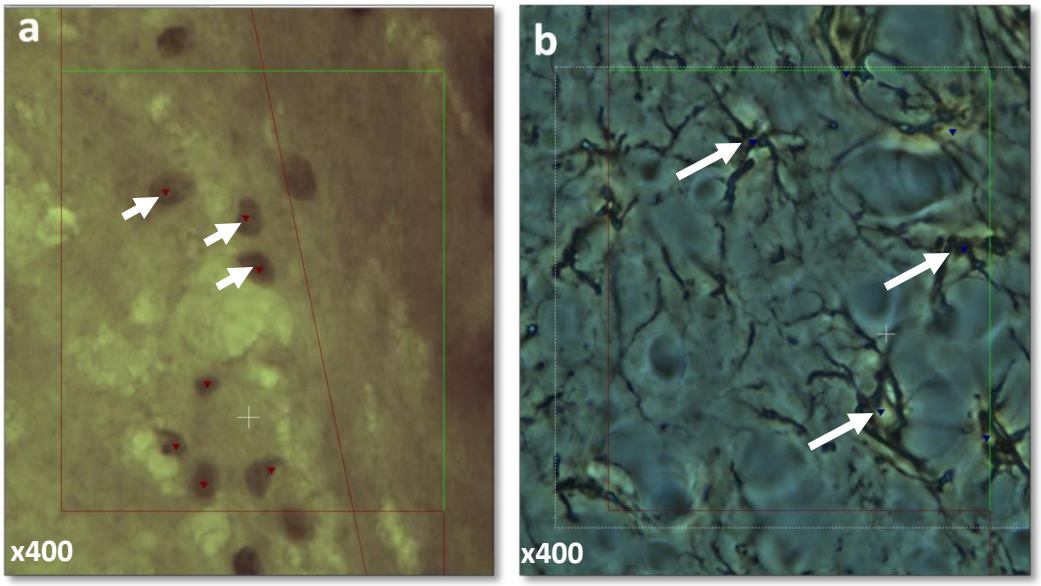

Figure 2.9 Image showing in process measurement of HX stained (a) and GFAP positive (b) cell counts using optical fractionator.

Arrows indicate selected HX stained cells (a) or GFAP positive cells (b) which underlie inside of the counting frame (green and red lines).

2.7.6 GFAP stained astroglia primary process length measurement using Neurolucida

Morphologic analysis was performed on 80 astrocytes in each animal (10 astrocytes per section). Neurolucida software (Version 9, MicroBrightField, Inc., Germany) was used to trace the boundaries of the ROI's and astrocytes. Astrocytes within the

ROI were traced in a systematic random manner, taking care to avoid those which were superimposed upon other astrocytes or blood vessels. The traced astrocytes were then analysed using Neurolucida explorer software (Version 9, MicroBrightField, Inc., Germany). Morphological analysis was performed by an observer who was blind to the experimental groups. Primary process length was measured using 100× objective (Nicon UPlan FLN, numerical aperture (NA)=1.30) and by counting the primary processes extending directly from the soma in both the lateral and central quadrants of astrocytes in the same sections.

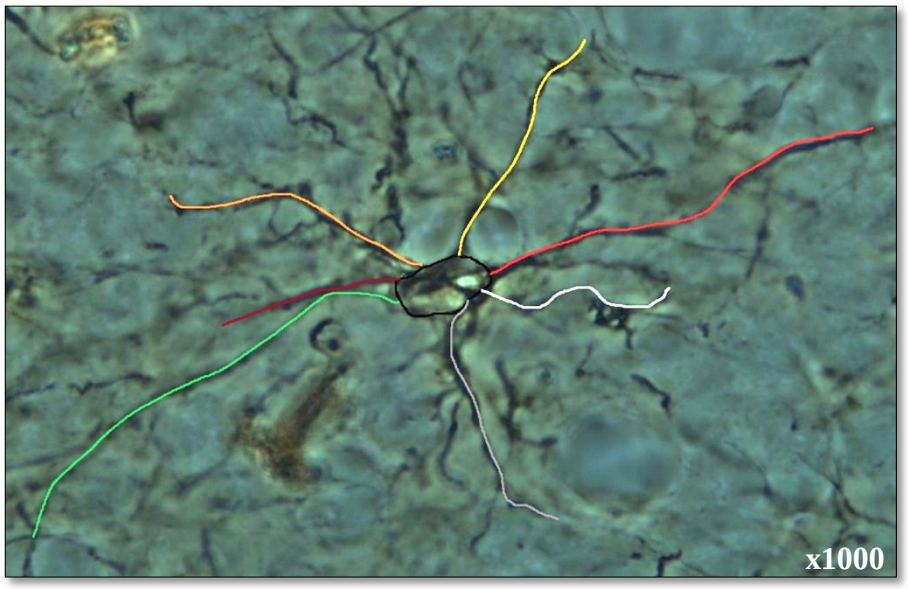

Figure 2.10 Image showing in process measurement of GFAP positive primary process length using neurolucida software.

In the above figure each primary process length is traced individually by focusing microscope. Once all the processes are traced the neurolucida tool provides estimate of the total length measured.

2.8 Quantitative autoradiography – Receptor analysis

Receptor autoradiography is a technique used to localise the radioactive ligands bound to specific receptors in the tissue. The energy emitted from the radioactive ligands or molecules collides with the nuclear emulsion or film due to which the distribution of the radioligand in the tissue is mapped on the film. Therefore, the autoradiogram obtained provides a detailed localisation of the specific targeted receptors of interest on the tissue subjected to the experimental condition. Autoradiography is a very sensitive technique which allows detection of the low levels of the receptors and is widely used in the histological studies to detect radiolabelled substances. Film autoradiographic technique consists of the following experimental steps: incubation of tissue with the selective and specific radioligand, exposure of films to apposed sections which contain traces of radioactivity retained in the tissue, film development and image analysis.

2.8.1 Experimental Design

A between-groups comparison experimental design was used to examine the potential effects of prenatal dexamethasone treatment on the distribution of the central oxytocin and 5HT-1A receptors in adult Sprague-Dawley rats. The sample (n=24) comprised of male and female rats and included two conditions (control and treatment, i.e. dexamethasone-treated in utero). The independent variables were gender (male vs. female) and condition (control vs. dexamethasone) of the subjects, while the dependent variable was the level of oxytocin and 5HT-1A receptor binding as determined by the autoradiography analysis in the ROIs.

2.8.2 Autoradiography Procedures

2.8.2.1 Oxytocin receptor – autoradiography protocol

- Selected brain sections (144 slides) were taken from the freezer (-80°C) and left at room temperature for 2 hours to thaw. The sections consisted of the cingulate cortex, prelimbic cortex, dorsal hippocampus, ventral hippocampus, hypothalamus, septum, nucleus accumbens and raphe nucleus as regions of interest (ROI).
- The slides were placed in two separate trays. Adjacent slides representing the same ROIs were designated for total and non-specific binding. As a result, 72 slides were marked for total binding and 72 slides for non-specific binding.
- [^{125}I] OVTA radioligand with specific activity of 2200 Ci/mol (ARI 0191, American Radiolabeled Chemicals, Inc.) was used for the oxytocin receptor labelling following Insel and Shapiro, 1992 protocol.
- The sections were incubated with 50 pM [^{125}I] OVTA in 50mM Tris-HCl (pH 7.4), 10mM $MgCl_2$ (Sigma-Aldrich, UK), 0.1% BSA (Invitrogen, UK), and 0.05% bacitracin for 60 minutes at room temperature for total binding. Controls for nonspecific binding were incubated with additional 1 mM cold oxytocin (Sigma-Aldrich, UK).
- Following, the incubation slides were quickly rinsed with washing buffer containing 50mM Tris-HCl with 0.1% BSA, 0.01% Triton X-100 (Sigma-Aldrich, UK), and 100mM choline chloride (Sigma-Aldrich, UK) to stop the binding reaction and were placed into slide holding racks.
- Each rack with slides then underwent washing rinses for 3x8 minutes in the washing buffer. Later, sections were quickly dipped in ice-cold distilled water.

2.8.2.2 5HT-1A receptor - autoradiography protocol

The autoradiography procedure was carried out according to Leventopoulos et al (2009).

- 144 polysine glass slides containing the appropriate sections were taken from the freezer (-80^{0}C) and placed onto plastic trays to thaw at room temperature for 2 hours prior to pre-incubation. In order to avoid confusion, the slides were separated in total binding and nonspecific binding groups.
- Meanwhile, all solutions and working reagents were prepared fresh (refer Appendix for details). Stock [^{3}H]WAY-100635 (Amersham Biosciences, UK) vial was taken out from the freezer (-20^{0}C) and left to reach room temperature before using.
- Slides pre-incubated in 50 mM Tris-HCl buffer, pH=7.5, at room temperature for 30 minutes. Approximately 0.5ml of solution was used to cover the sections on each slide.
- The pre-incubated buffer was removed from the slides and the remainder was blotted with filter paper in order to avoid over dilution for the next step.
- The sections were incubated in 50 mM Tris-HCL buffer, pH=7.5 containing 10µM pargyline (Sigma-Aldrich, UK) and 2nM [^{3}H]WAY-100635 radioligand for 2 hours at room temperature. In order to determine non-specific binding, 10µM 5-HT (Sigma-Aldrich, UK) (Serotonin hydrochloride) solution was incubated into an adjacent (serially cut) sections for similar period.
- After incubation with the radioligand and the block, the slides were placed inside small plastic microscope rack trays and dipped 3 times in ice cold buffer, in glass containers.

- Then, the sections were washed 2x2 minutes in ice cold Tris-HCl buffer, pH=7.5.
- Finally, the sections were quickly dipped in 4^0C distilled water three times, and left overnight to dry under an air stream.

2.8.3 Exposure stage

All the radiolabelled and blocked slides were placed in a specific manner inside the cassette where total and nonspecific binding slides were placed adjacent to each other in same cassette. Empty space was covered with blank slides. Before placing the slides, 2-3 layers of paper were cut at the same size as the film and were placed on top of the film. Standards were also placed inside the middle of each cassette. All cassettes containing the appropriate slides were transferred from the radioisotope lab to the dark room for exposure. All sources of lights were covered, room lights were switched off and a DL10 safelight (Ilford Imaging Ltd) was used. One hyperfilm was then carefully taken and placed onto the slides with the emulsion side of the film facing the slides and shinny side upwards. To further check the correct direction of the film the notch (cut) on the film was on the top left corner of the cassette.

The cassette was firmly closed with 3-4 layers of paper placed on top of the film. The cassettes were additionally wrapped around with silver foil for further light protection, sealed and labelled accordingly. All the cassettes were finally placed into a black colour bag and were stored in cool and dry place for the remaining exposure duration.

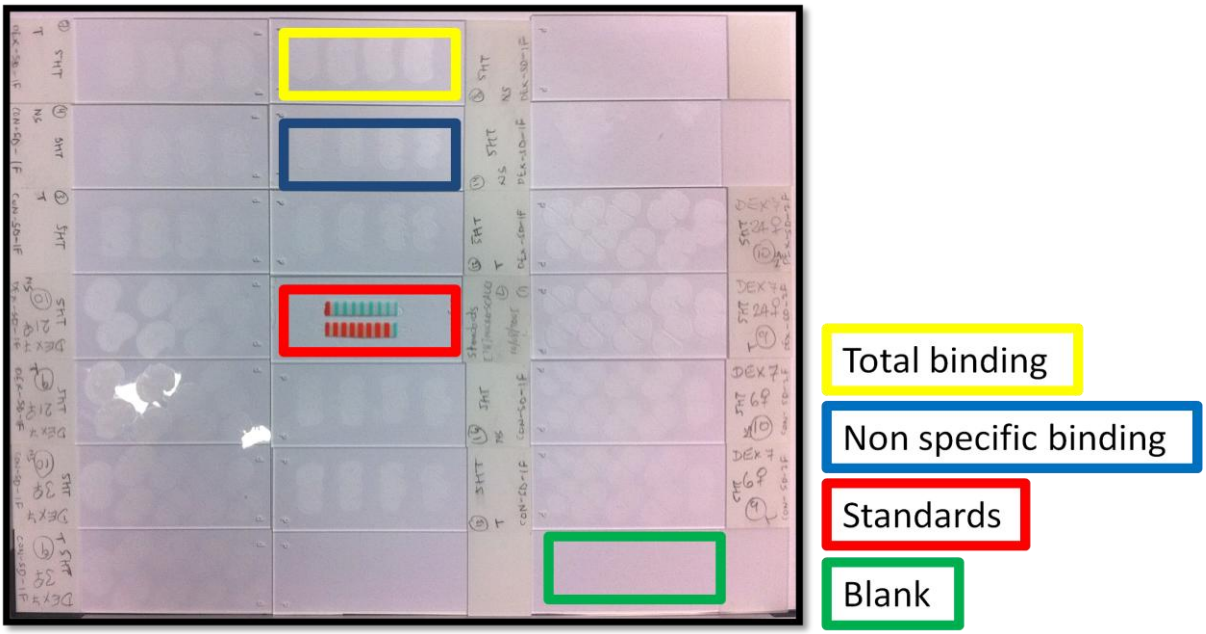

Figure 2.11: Photograph of the slide layout inside the hypercassettes during and after exposure.

The above image represents the placement of one set of the blank, total binding, nonspecific binding and standard slides in a hypercassette which is ready for film exposure.

2.8.4 Film development

After the incubation period forty eight hours for oxytocin and seven weeks for 5HT-1A, film development was carried out in the dark room of the laboratory at Roehampton University. First, three solutions were prepared: Developer (Kodak ™ processing chemicals, Sigma Aldrich, UK), Fixer (Kodak ™ processing chemicals, Sigma Aldrich, UK) and Distilled water with a drop of acetic acid.

Each film was taken off the hypercassettes carefully and was placed into the developing tray with the shiny slide down for one minute with slight agitation ensuring the film is completely dipped in the solution at all times. Next, the film was

removed and placed directly into the distilled water mixed with a drop acetic acid again for 1 minute and later was placed in fixer solution for three minutes with constant agitation. During the procedure, extreme care was taken to ensure that the film was not in contact with the bottom of the tray, which otherwise might have caused damage or scratch to the film affecting its quality. The films were then transferred into the cold running water tray for 30 minutes. Finally, the films were hung up and left to dry out at room temperature overnight. The sample of these developed films is shown in the Figure 2.12, below.

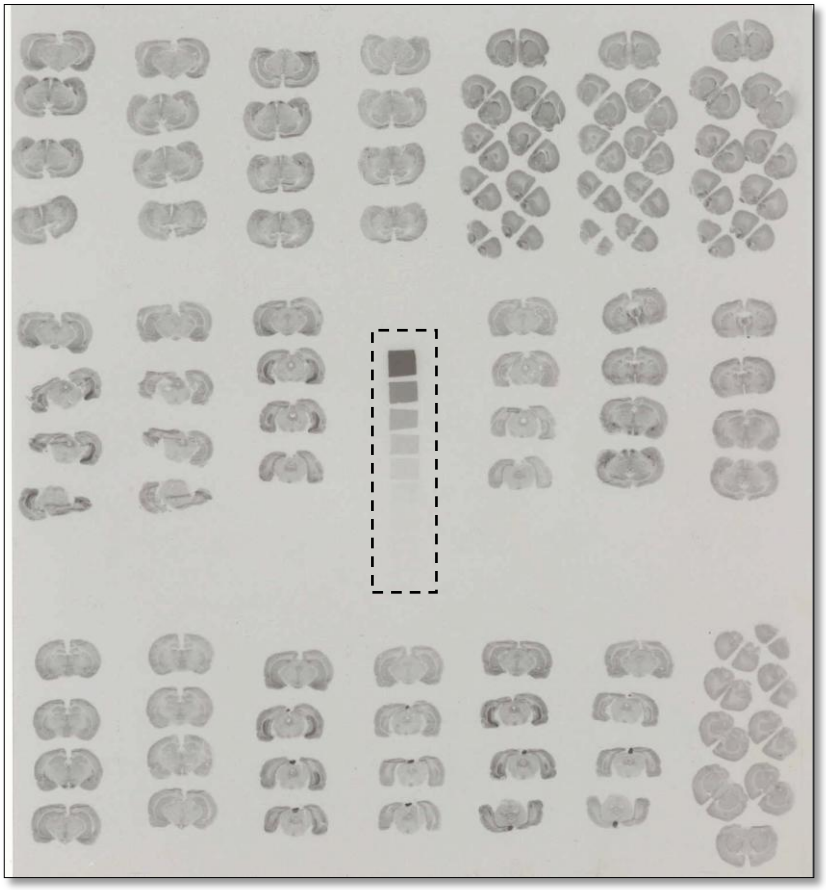

Figure 2.12 Image of the developed film exposed to oxytocin receptor radioligand.

It shows different brain sections representing various ROIs and microscale standards.

2.8.5 Image analysis using MCID

The MCID™ Basic (Version 7.0, Interfocus imaging research Ltd, England) image analysis platform system was used for quantitative densitometry of the autoradiographic films. Images were captured by means of a lightbox-desktop illuminator (Northern Light Precision Illuminator) and a digital colour video camera (CoolSNAP™cf, Photometrics). The programme provides data into ligand binding per unit of tissue mass (for example fmol/mg tissue weight) or isotope concentrations (for example nCi/mmol tissue weight). Before sampling and analysis of the films the MCID™ system needs to be calibrated and adjusted. The procedure used in order to analyse all of the autoradiographic films and images was as follows:

2.8.5.1 Magnification and focus adjustments

The light box was left to warm up for 10 minutes. Room lights were switched off and the prepared autoradiographic film was placed on the lightbox with the shiny side upwards, in order to adjust the magnification and focus. The image was digitized and magnification was obtained by moving the height of the camera at the point where the section image was inside the view field. The numerical aperture and focus was adjusted at which a clear focused image was generated.

2.8.5.2 Flat field corrections

In order to perform the accurate densitometry, the uneven illumination or shading error over the field of view, something typical with the camera generated images, was corrected for using the MCID™ FFC tool. First, the film was removed from the field of view and a pseudocolour image was presented by pressing F2. The lightbox

illumination intensity was adjusted so that the background appeared bluish-pink. The FFC was then performed following the MCID™ manual guidelines (www.mcid.co.uk).

2.8.5.3 Distance calibration

Distance calibration was performed using a glass scale calibration reticule.

2.8.5.4 Density calibration

Density calibration was done in order to express the autoradiographic data in units of an isotope or ligand concentration. To achieve this, a set of isotope standards was co-exposed with the sections. A calibration table is shown below. The standard values were created by entering the list of standards provided by the supplier. The standard values were corrected for the decay factors as applicable. A standard curve was constructed which converts relative optical density to standard values (for example, nCi/mg). It was always ensured that during sampling a homogeneous area was covered. Either linear or 3rd degree polynomial curve was chosen as appropriate and calibration file was finally saved.

(A)

No	Standard Value	*0.726 (correction value)
1	0.931	0.68
2	1.457	1.06
3	2.18	1.58
4	2.193	1.59
5	3.32	2.41
6	3.444	2.50
7	5.304	3.85
8	5.93	4.31
9	8.316	6.04
10	13.6	9.87
11	21.088	15.31
12	33.55	24.36

(B)

No	Standard Value
1	93
2	46
3	23
4	11.5
5	5.7
6	2.8
7	1.4
8	0.7
9	0.35
10	0.18

Table 2.2 - Tables of the standard calibration micro-scales for (A) - tritium [^{3}H] and (B) - iodine (I^{125}) radioligands

2.8.5.5 Sampling

Before sampling, all sections of the film were digitised and saved with their calibration curve (Figure 2.13). In all the sections, the ROI was identified using rat brain atlas (Paxinos and Watson, 2005). A specific sampling grid was designed and allocated to each ROI. Both right and left hemisphere was sampled from each section. Values were estimated in nCi/mg and were converted into fmol/mg. The mean value of the left and right hemisphere data was calculated and was used for statistical analysis.

2.8.6 β counting

In order to know if the radiologand dilutions were correct prior to the autoradiography, 10µl of the working solutions (total binding and block) used in the incubation step was added to scintillation vials and labelled. Then 5ml of the scintillation cocktail was added to all vials using the dispenser. All vials were thoroughly mixed. The scintillation vials were placed inside the β-counter (Beckman Coulter^{TM}, USA) and was counted using a program for either beta (Tritium) or gamma (125-Iodine) radiation.

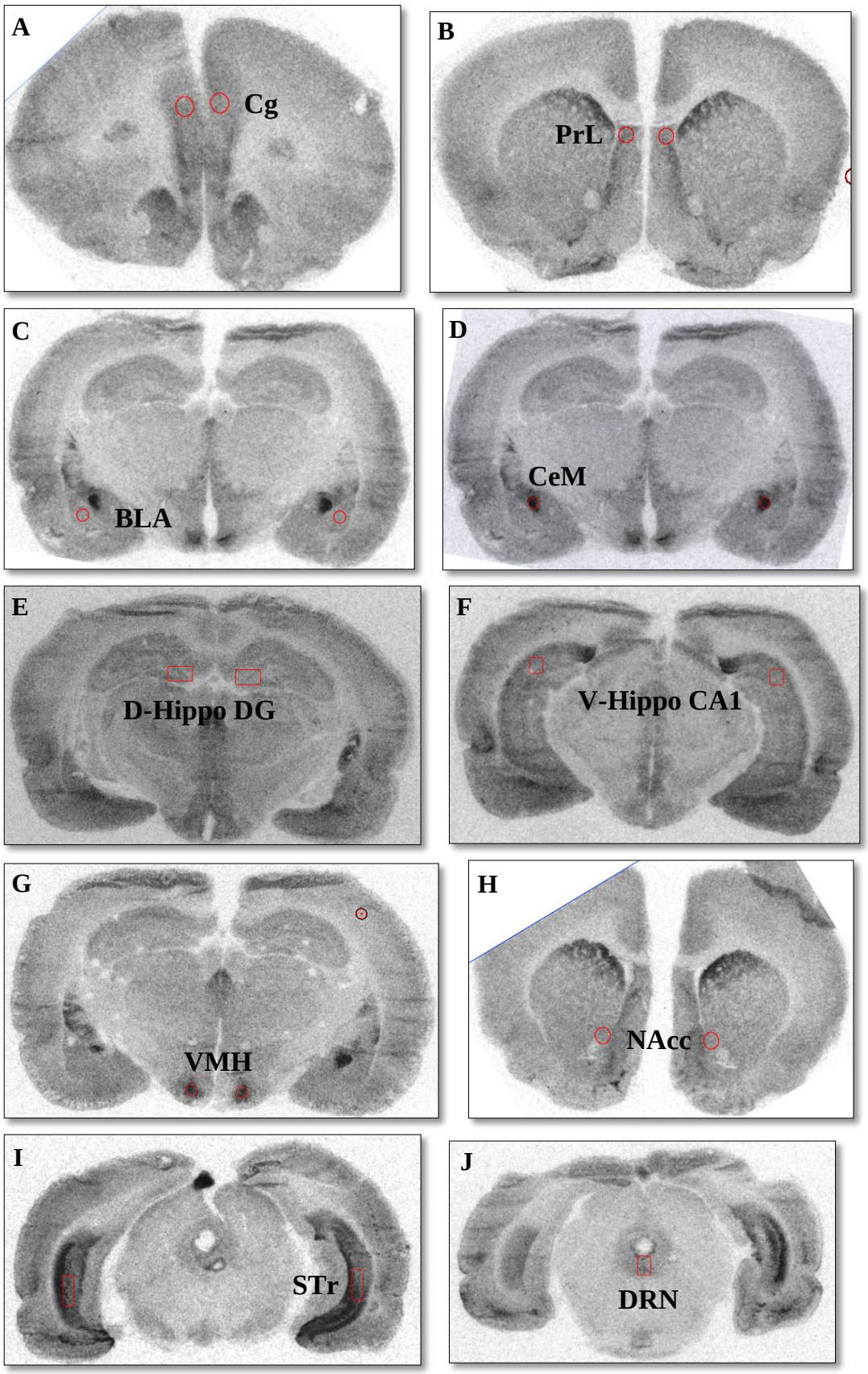

Figure 2.13 Representative autoradiographic image of rat brain coronal sections showing sampling grid (red shaped) used for receptor binding quantification of the region of interest during sampling.

Cg – cingulate cortex, PrL – prelimbic cortex, BLA – basolateral amygdala, CeM – central amygdala, D-Hippo - dorsal hippocampus, DG - dentate gyrus, V-Hippo – ventral hippocampus, CA1 - coruna ammonis 1, VMH – ventromedial hypothalamus, Nacc – nucleus acumens, STr – subiculum transition area, DRN – dorsal raphe nuclei.

2.9 *In vitro* neural stem cell procedures

2.9.1 Neural stem cell cultures (NSC)

The NSC proliferation media were freshly prepared prior to cell trypsinisation. It comprised of 49ml D-MEM/F12 containing L-glutamine and was further supplemented with 1ml B27 supplement minus the Vitamin A hormone mixture and combination of 30µl of growth factors (Growth factors: 20µl of 20ng/ml fibroblast growth factor (FGF) and 10µl of 20ng/ml epidermal growth factor (EGF)). Media was stored in the Heracell 150i CO_2 incubator (Thermo Scientific, UK) with temperature and CO_2% parameters set to 37^0C and 5% of BSA-1% and BSA-4% were prepared on the day using original 7.5% stock solution. The 7.5% BSA was diluted with Lebovitz's L-15 medium (Gibco-Invitrogen).

2.9.2 Isolation of E16-18 mice cortex cells – preparation of neural stem cells for *in vitro* cultures

NSC (kindly provided by Dr. Molina-Holgado, Stem Cell Bank, University of Roehampton, UK) were isolated from cortex dissection at gestation day E16-18 from C57BL/6J (Wild type – WT) mice (Harlan, UK) (as described in Reynolds and

Weiss, 1992; Molina-Holgado et al, 2007), where day E0 was the day a gestational plug formed. Likewise, primary neurospheres were generated as per the above published protocols. These cells were then grown in untreated T75cm^2 Nunclon flasks (Nuc, 156499, Roskilde, Denmark) for 7-10 days. Subsequent to this period, cells were passaged by mechanical dissociation to acquire secondary cultures.

All the animal care procedures and their handling were in accordance with the European Council instruction guidelines (86/609/EEC), the Home Office Animals Scientific Procedures Act, 1986 (Schedule 1, UK) and procedures conducted in agreement with the European Union Legislation (86/609EEC). The study handled generation of NSC originating from the cortex, hence it was possible to generate neuronal as well as non-neuronal lineage.

2.9.3 Recovery of cryo-preserved neurospheres and their preparation for further sub-culturing

Neurospheres were spun down in a universal and then re-suspended in 0.5ml 10% DMSO-containing media. Thereafter, cells were immediately placed into liquid nitrogen, which enabled long term storage. Prior to use, cryovials containing frozen NSC were removed from the liquid nitrogen. Each cryovial contained 0.5ml of NSC sample. Cryopreserved neurospheres were then rapidly thawed. 1ml of fresh pre-warmed media (Dulbecco's Modified Eagel Medium: nutrient mixture F12) was immediately added to each vial and was mixed gently. As the cells began to thaw, samples were then transferred into the pre-warmed media (15ml conical falcon centrifuge tubes, BD Facon^{TM}, BD Biosciences) were then defrosted completely. 8.5ml media was later added into the WT strain yielding a final volume of 10ml. Following this, samples were centrifuged (sigma laborzentrifugen) resulting in a

pellet. The supernatant, containing 10% dimethyl sulfoxide (DMSO) was aspirated and pellets were re-suspended in 10ml of proliferation NSC media, comprised of DMEM F-12 with L-glutamine (Gibco) supplemented with B27 hormone mixture (in house), 20ng/ml of FGF-2 (Recombinant Human, Peprotech 100-18B) and 20ng/ml of EGF (Murine, Peprotech 315-09). NSC were incubated at 37°C, 5% CO_2. Primary culture was maintained for 7-10 days. A secondary culture was then generated by single cell sub-culturing: the single cell sample was then mixed thoroughly and transferred into untreated T75cm^2 Nuclon flasks (Roskilde, Denmark) allowing neurospheres to grow (7-10 days). The initial experiments were carried out after 3-7 passages.

Since cells were previously frozen additional time to reactivate their mitotic processes was required. The cell forming neurospheres were supplemented (every 3-4 days) with a 6µl mitogenic factors mixture (10µl EGF and 20µl FGF) per flask.

The present study commenced at sub-culturing the ninth generation of WT – NSC strain. Throughout the study, NSC were sub-cultured and passaged further; the study ended at WT – 11th generation.

2.9.4 Generation of secondary neural stem cells

Prior to serial dilution assays and cell challenges with drugs, formed clusters were passaged when they reached 60-70% confluence (approximately 5-7 days after initial plating). Evaporation of NSC proliferation media was calculated at 10% per 7 days.

Neurosphere – containing media of WT strain were collected. Firstly, the flasks was gently shaken to detach the attached neurospheres. Then the bottom of the flask was

rinsed several times to collect as many detached cells as possible. Thereafter, the medium containing the floating neurospheres was transferred to 50ml tube and centrifuged. Subsequent to centrifugation, a pellet for each strain was formed.

The supernatant was then aspirated and 1ml 0.05% trypsin/EDTA (Gibco) was added to pellets of each strain to dissociate clusters of cells and produce a single cell suspension. The solution was gently disaggregated and aggregating cells previously forming neurospheres were separated. The samples were then placed in the incubator and the digestion reaction was allowed for 10 minutes. However, at eight minute, samples were disaggregated and at the ninth minute the trypsinisation reaction was stopped by adding 2-3ml of 4% BSA solution to each strain containing 50ml Falcon tube. The volume was then adjusted to a level of 10ml using 1%BSA; this subsequently blocked trypsinisation. In order to isolate the single cells that had disaggregated, both samples were centrifuged. The supernatant was aspirated and pellets were supplemented and re-suspended in 10ml NSC proliferation media containing supplements and growth factors. Medium – cell mixture samples were then filtered through at cell strainer (BD Biosciences, UK) in order to remove all debris that could potentially interfere with cell proliferation. Further, subsequent to several strokes, the number of NSC/ml per each strain was determined (special cell counter divided into nine main squares, where each square contained nine mini squares), using an inverted microscope and manual cell counter (Tamaco). Cells were then finally plated into T75 flask at a density of 100,000 cells/ml.

2.9.5 Assessment of cell death (LDH)

Cytotoxicity was evaluated by release of the cytosolic enzyme lactate dehydrogenase (LDH) into the culture medium by dead and dying cells (CytoTox-96 LDH assay, Promega, Southampton, UK). Total LDH release was calculated by incubating untreated cells with 0.1% Triton X-100 for 10 min (37^{o}C, 5% CO2, 95% air) to induce maximal cell lysis. Absorbance was measured at 490nm. Treatment values were then expressed as a percentage of the total LDH release. Background LDH release (media alone) was subtracted from the experimental values. (Molina-Holgado et al., 2008)

2.9.6 Differentiation Protocol

To evaluate the effects of dexamethasone on neural stem cell differentiation of WT strain this experiment was performed. Initially, coverslips were coated with 50µg/ml poly-ornithine in water for overnight at room temperature. The following day washing with distilled water was done twice for 2 minutes each. Then, coverslips were coated with 5 µg/ml laminin in PBS for overnight at room temperature. Washing with PBS was done for 5 minutes. Plate cells (20,000/well) were then incubated in NS-A media with 10ng/ml EGF/FGF. Then, the media were replaced to NS-A with 5ng/ml FGF and B27. After two days dexamethasone (Sigma Aldrich, UK) was added and maintained for 48h. Finally cells were fixed using 4% paraformaldehyde (PFA) solution and then the coverslips were used for immunocytochemistry procedures.

2.9.7 Immunocytochemistry

Firstly, plate neurospheres were directly placed onto coverslips (coated for 4-6 h with poly-D-Lysine (Sigma Aldrich, UK) for 1hr or, alternatively, cells were mechanically dissociated at a density of 30,000 cells/cm^2 for 1-4 hr. This was followed by cell fixation procedure using 4% paraformaldehyde for 25 min. Rinsing was then done using PBS buffer for 2 x 5 minutes. Overnight incubation with primary antibodies, i.e. GFAP (1:500) and S100 (1:400) was done at 4°C. Immunostaining vehicle solution used was PBS + 0.1% Triton X-100 + 2% NHS.

Next day, the first step was washing with PBS solution for 2 x 5 minutes. Then, secondary antibody (anti-mouse with TritC – 1:200 and anti-rabbit with FITC – 1:200) in immunostaining vehicle solution were incubated for 1 hr. This was followed by PBS washing 2 x 5 minutes and with water 1 x 5 minutes. The coverslips (cells upside down) were then carefully removed from the wells using forceps and were mounted on plain glass slides with a drop of mounting medium with DAPI (Vecatshield®, Vector Lab) in order to visualize cell nuclei. Nail polish was used to further seal the edges of the coverslip on glass slide (Rubio-Araiz et al., 2008). After air drying for 30 minutes in dark, cells were analysed using fluorescent microscope (BX51, Olympus) and View Finder 3.0.1 imaging software (Pixera corporation, USA).

2.10 Statistical analysis

All the statistical analysis was performed using the Statistical Package for Social Sciences (SPSS version 17.0, Inc., USA). Preliminary analysis was undertaken to show that data were normally distributed (Kolmogorov–Smirnov test) and that variances were equal (Levene's Median test). One-way ANOVA with a post hoc Tukey test was performed, comparing between group and within group differences (eg. NH vs EH vs ED). Two-way ANOVA was used to find interactions between variables (eg. Sex x Treatment). Independent t-test was performed to measure the difference between two groups (eg. Control vs Dex treated). Pearson correlation test and linear regression were used to find relationships between brain weight and volume (eg. pQCT analysis). Statistical significance was set at $p<0.05$. Results are expressed as mean values ± SEM.

Chapter 3

Early postnatal manipulation: effects of early deprivation on brain volume and astroglia

3.1 Introduction

As indicated in Chapter 1, early life environment plays a critical role in the regulation of brain and behaviour (Levine, 1960; Newport et al., 2002; Pryce and Feldon, 2003). In mammals, the mother – infant relationship plays a key role in the development of the offspring where mother provides thermal, somato-sensory, olfactory, visual and auditory stimulation, which are essential for postnatal development (Pryce, 1996). Clinical and preclinical studies have shown the importance of early life environment and adversities caused by early life stress, such as social neglect, loss of parents, abuse, which leads to neurobiological and psychiatric disorders in adulthood (Heim and Nemeroff, 2001; Kaufmann et al., 2000).

Animal models with disrupted maternal care may result into depression like behavioural phenotypes (Rüedi-Bettschen et al., 2006). In preclinical approach, it is possible to model a relation between early life stress and depression by early deprivation (ED). Such experimental modelling reflects similar human traits such as neglect like behaviour and reduced motility towards reward (Pryce et al, 2005) and results in long-term neurobehavioral changes (Pryce et al., 2002a). Studies have shown reduced 5-HT_{1A} receptor binding (Leventopoulos et al., 2009), reduced CA-hippocampal field volume (Lehmann et al., 2002) and reduced astroglial density (Leventopoulos et al., 2007) in response to early life manipulation in the form of deprivation.

The recent research suggests a role for an epigenetic mechanism in maternal care disruption, which influences the development of the offspring (Weaver et al, 2004; Murgatroyd et al., 2009; Murgatroyd and Spengler, 2011). However, it is estimated

that a 40-50% chance of having a mood disorder such as depression is genetic (Nestler et al, 2002). In the present study, two rat strains of different responsiveness to ED: Wistar (showing neuro-behavioural responses to ED) and Lewis strain (resistant to ED) were investigated (based on personal communication, Dr. C Pryce) (Kosten and Ambrosio, 2002). This helps to assess the power of environmental effects against genetic trends.

A series of studies have implicated the role of HPA axis alteration in stress related vulnerabilities (Heim and Nemeroff, 2001; Heim et al., 2004; Pryce et al., 2011) and the hippocampus, the regulator of HPA axis and involved in learning and memory, is the region sensitive to stress and stress hormones and has abundant glucocorticoid and mineralocorticoid receptors (de Kloet et al., 2005) which suggests its possible involvement in stress disorders.

Hippocampal volume losses have been observed in patients suffering with posttraumatic stress disorder (e.g. Bremner et al., 1995; Apfel et al., 2011), major depression (e.g. Bremner et al., 2000; Sheline et al., 2003) and schizophrenia (Heckers, 2001). Clinical reports of significant volume losses in patients suffering from childhood sexual abuse (Sapolsky, 2000) and neglect (Teicher et al, 2004) also add to the early life stress vulnerability. Meta-analysis by Viderbech and Ravnkilde (2004) concluded hippocampal volume reduction on average by 8% in the left and 10% in the right hemisphere in patients suffering from depression. Even preclinical studies have also reported stress induced hippocampal vulnerability in the tree shrews (Ohl et al., 2000; Czeh et al., 2006), macaque (Jackowski et al., 2011) and rodents (Lehmann et al., 2002; Czeh et al., 2010). These results clearly suggest early life stress, possibly via hippocampal dysfunctioning, leads to the development of

stress related disorders in later stages of life (Heim and Nemeroff, 2001; McEwen, 2003).

It should be noted that hippocampal plasticity is a well-replicated finding in stress related disorders (Pryce et al., 2011). However, other brain limbic regions such as amygdala, prefrontal cortex are also involved and affected by neurological disorders. MRI studies carried out in depressed patients have shown a reduction in the amygdala (Sheline et al, 1998) and hippocampus (Frodl et al., 2010) whilst another study reported an increase in the amygdala volume in both men and women suffering from depression (Bremner et al, 2000).

Until now most of the human and animal studies hypothesised various possibilities supporting hippocampal volume loss such as dendritic remodelling, death of neurons due to excess of glucocorticoids (Sapolsky, 2000) and loss of CA3 pyramidal cells (Sapolsky, 1985). It should also be noted that no stereological quantification was performed in the mentioned studies. Currently, unbiased stereological cell counting, volumetric and morphological techniques are the method of choice for evaluating changes in specific brain regions.

Volume reductions in the hippocampus suggest plastic rearrangements within this brain subregion and hence depression with its hippocampal atrophy is now recognised as a disorder of neuroplasticity (Mc Ewen, 2001 and 2005; Duman 2002a, 2002b; Duman 2004; Fuchs et al., 2004; Czéh and Lucassen, 2007). A decrease in the rate of neurogenesis in the adult hippocampal dentate gyrus has also been considered as a causative factor in hippocampal shrinkage (Henn & Vollmayr, 2004). Various animal studies suggest that stress and glucocorticoids are among the potent inhibitors of neurogenesis in the adult dentate gyrus (Gould et al, 1997; Czeh et al,

2002; Mirescu & Gould, 2006). However, most of the research studies have focused primarily on neurones and less attention has been given to glial cells, which markedly outnumber neurones in the brain (Joelving et al, 2006) and constitute a major population of the hippocampal volume fraction. And due to this, change in glial number or morphology can significantly affect hippocampal shrinkage (Czeh & Lucassen, 2007).

Recent clinical studies have demonstrated a reduced ratio of glia per pyramidal neurone numbers, consistent with a reduction in the glial cells in major depressive disorder patients (Cobb et al, 2006); reduced GFAP stained astroglial cells in hippocampus have been reported by Muller et al (2001). Reduced astroglial GFAP-immunoreactivity has also been found in the prefrontal and cingulate cortex, basolateral amygdala and hippocampus (Leventopoulos et al, 2007). There is growing evidence that astroglia, as defined by their selective marker GFAP, play a role in the pathophysiology of depression (Harrison, 2002; Miguel-Hidalgo et al (2000); Müller et al (2001); Bowley et al., 2002; Rajkowska and Miguel-Hidalgo, 2007) and stress-related and depression-like conditions in animal models (e.g. Czéh et al., 2010; Leventopoulos et al. 2007, Banasr and Duman, 2008; Banasr et al., 2010).

3.1.1 Aims and Hypotheses

The previous study findings support the view that early life stress modulates hippocampal functioning and thus it is of relevance to study experience - dependent hippocampal remodelling. The present study investigates the ED effects on the regional brain volumes and astroglial morphology using the stereology quantification technique. It is hypothesised that ED leads to reduction in the volume of the hippocampus and impoverishment of astroglial morphology in Wistar rats. The specific hypotheses of the present study were as follows:

- ED will result in a reduction of the volume of hippocampus, amygdala and nucleus accumbens without affecting total cell density.
- ED will be associated with reductions in GFAP positive astroglial cell number and primary process length.

3.2 Results

3.2.1 Brain Weight and Volume

No significant treatment effects on the brain weight were observed in both Wistar [F (2, 15) = 1.662, p= 0.223] and Lewis rats [F (2, 15) = 1.862, p= 0.211]. A significant difference was observed between the two strains [F (1, 24) = 4.302, p< 0.05] and no change was seen in strain vs treatment [F (2, 24) = 0.628, p = 0.542].

		NH	EH	ED
Wistar	Brain Weight (mg)	2020	1920	1910
	Brain Volume (mm^3)	1986.4	1978.5	1908.7
Lewis	Brain Weight (mg)	2056	2080	2003
	Brain Volume (mm^3)	2032.9	2105.3	1974.3

Table 3.1 - Whole brain weight (g) and brain volume (mm^3) as measured by means of pQCT. Data are expressed as mean values.

The pQCT analysis indicated no significant treatment effects on the whole brain volume in Wistar rats (One-way ANOVA with post hoc Tukey test, F (2, 15) = 1.380; p= 0.282, ED vs NH (p = 0.314), ED vs EH (p= 0.388) and EH vs NH (p= 0.987) (Table 3.1).

However, significant change was seen between ED vs EH (p< 0.05) in Lewis rats [F(2,9) = 5.968, p< 0.05], ED vs NH: 0.260, EH vs NH: 0.460. Changes were also seen between strain - Wistar vs Lewis [F (1, 24) = 4.745, p<0.05], NH (p= 0.688),

EH (p= 0.029), ED (p= 0.121). No significant change was observed in strain vs treatment [F (2, 24) = 0.551, p = 0.583].

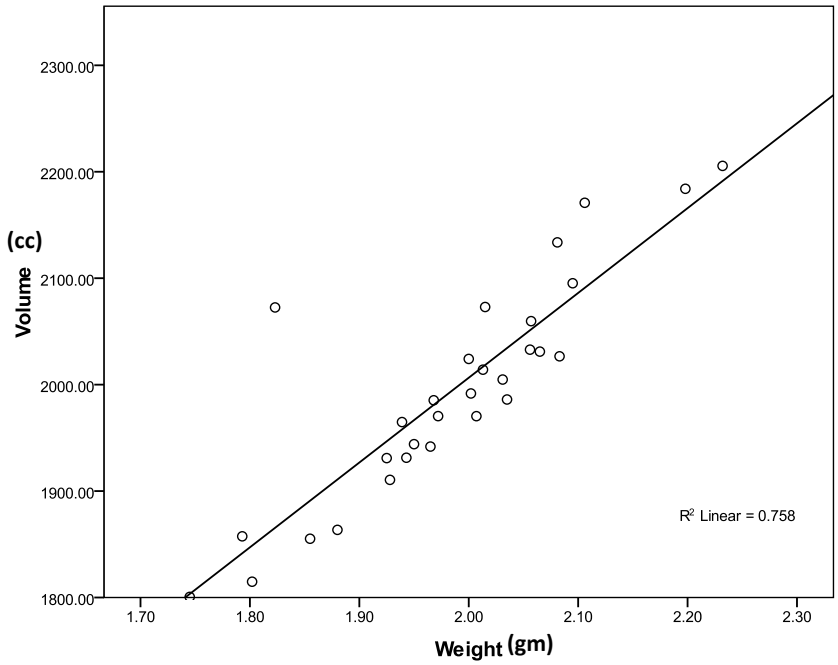

Figure 3.1 – Scatter plot showing the strong positive linear relationship between brain weight and volume (n=30).

In the course of the method validation a strong positive linear correlation and regression demonstrated a significant positive relationship between brain weight and volume (F (1, 28) = 87.635, p<0.001, R^2 = 0.758) (Pearson correlation, Figure 3.1). The whole brain volume analysis was highly reproducible with coefficient of variation of 0.91% and average measurement error of 0.662%.

3.2.2 Dorsal Hippocampal volume

For dorsal hippocampus volume, estimated by means of stereology with Cavalieri principle, there was a significant effect of Group ($F(2, 15) = 20.422$, $p < 0.001$) (Figure 3.2, Table 3.2). Volume was significantly decreased in ED vs NH rats (mean decrease of 12.5%, $p<0.001$) and in EH vs NH rats (7.5%, $p<0.01$). The reduction in ED vs EH rats (5.5%) was borderline non-significant ($p = 0.054$). There was a significant effect of Group on dorsal hippocampus volume as a percentage of whole brain volume ($F(2, 15) = 4.417$, $p<0.05$) with this parameter being significantly reduced in ED vs NH rats (9.0 %, $p<0.05$). No significant changes were observed in dorsal hippocampal volumes in Lewis rats (absolute = $F(2, 11) = 0.763$, $p = 0.490$ & relative = $F(2, 9) = 0.778$, $p = 0.488$).

There was no significant strain effect observed [$F(1, 26) = 0.075$, $p = 0.786$] and interestingly significant strain vs treatment effect was seen [$F(2, 26) = 4.573$, $p< 0.05$]. There were no significant differences between the left and right hemispheres in both Wistar and Lewis rats.

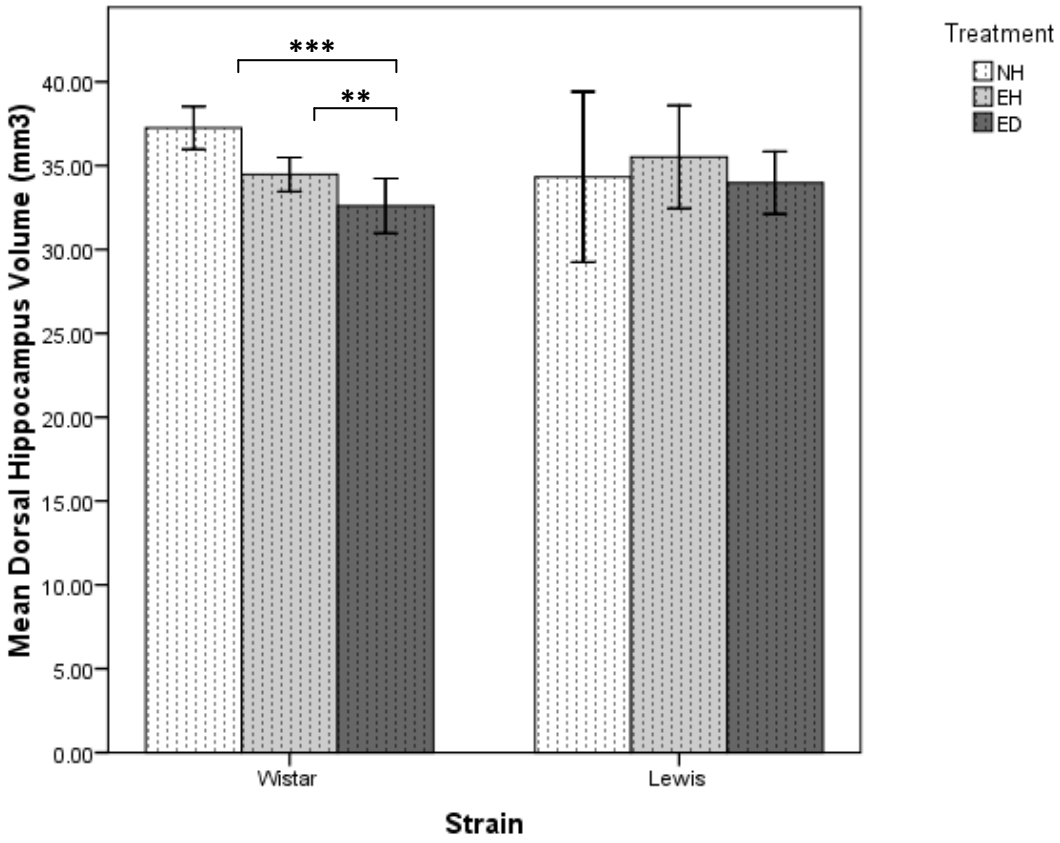

Figure 3.2 - Effects of early deprivation on the dorsal hippocampal volume in adult Wistar and Lewis rats.

Data presented as mean ±SEM, n=6 per NH, EH and ED in Wistar's and n=3 NH, n=5 EH and n=6 ED in Lewis rats (**p<0.01, ***p<0.001)

Absolute volume values(mm3)			
	NH	EH	ED
WISTAR			
Dorsal Hippocampus	37.25 ± 0.50	34.47 ± 0.39	32.6 ± 0.63 ***# **Δ
Basolateral Amygdala	0.93 ± 0.02	0.91 ± 0.03	0.88 ± 0.02
Nucleus Accumbens	2.67 ± 0.1	2.75 ± 0.13	2.71 ± 0.09
LEWIS			
Dorsal Hippocampus	34.3 ± 1.2	35.5 ± 1.1	34 ± 0.7
Basolateral Amygdala	0.94 ± 0.02	0.93 ± 0.01	0.93 ±0.01
Nucleus Accumbens	3.15 ± 0.09	3.11 ± 0.05	3.06 ± 0.08

Table 3.2 - Brain regional volume analysis – Hippocampus, Amygdala and Nucleus accumbens.

Data presented as mean ±SEM, n=6 per NH, EH and ED in Wistar's and n=3 NH, n=5 EH and n=6 ED in Lewis rats.

*p<0.05, **p<0.01, ***p<0.001, # vs NH, Δ vs EH

3.2.3 Basolateral Amygdala and Nucleus Accumbens volume

There was no significant effect of Group on the volume of the basolateral amygdala in both Wistar ($F(2, 15) = 1.458$, $p = 0.264$) and Lewis rats [$F(2,11) = 0.223$, $p = 0.804$]. No strain [$F(1, 26) = 3.746$, $p = 0.064$] and strain vs treatment [$F(2, 26) = 0.616$, $p = 0.548$] effects were observed in basolateral amygdala (Figure 3.3).

The nucleus accumbens volume also showed no changes in both Wistar ($F(2, 15) = 0.137$, $p = 0.873$) and Lewis rats [$F(2, 11) = 0.375$, $p = 0.696$] (Figure 3.4). However, significant strain effect was seen [$F(1, 25) = 25.316$, $p < 0.001$] with no strain vs treatment effect [$F(2,25) = 0.248$, $p = 0.782$].

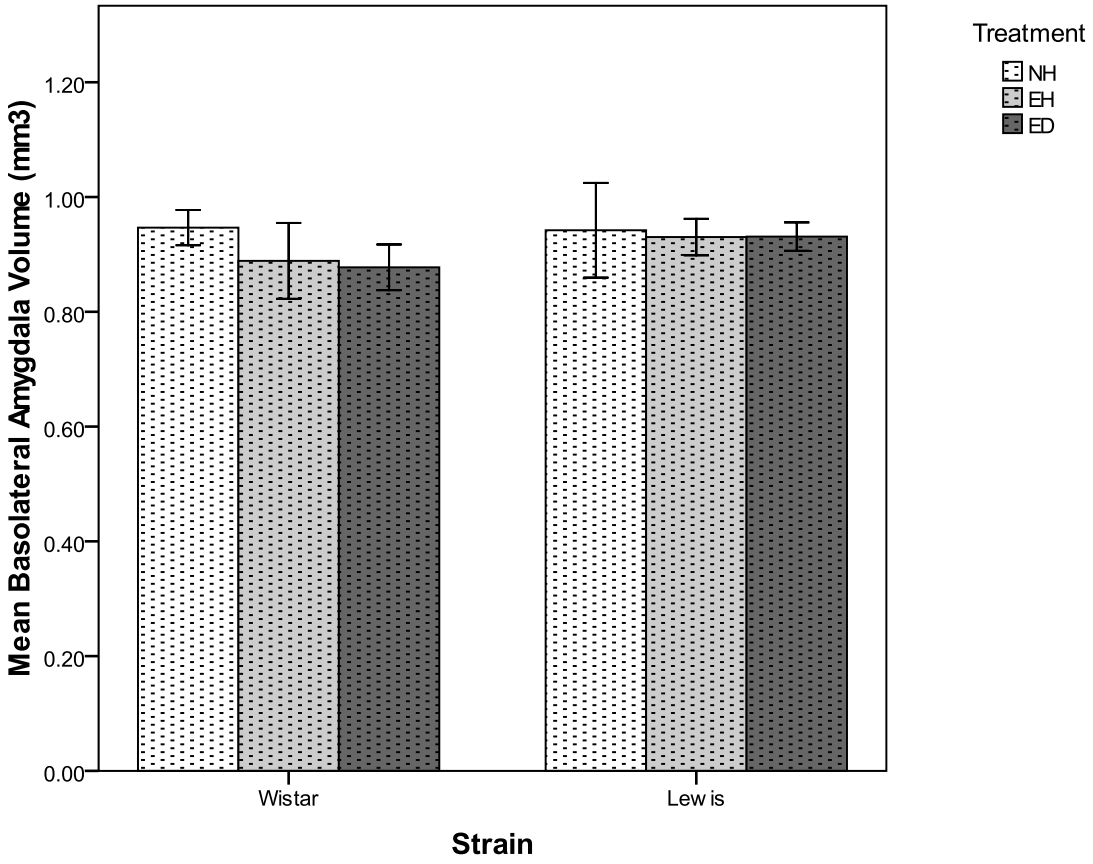

Figure 3.3 - Effects of early deprivation on the basolateral amygdala volume in adult Wistar and Lewis rats.

Data presented as mean ±SEM, n=6 per NH, EH and ED in Wistar's and n=3 NH, n=5 EH and n=6 ED in Lewis rats.

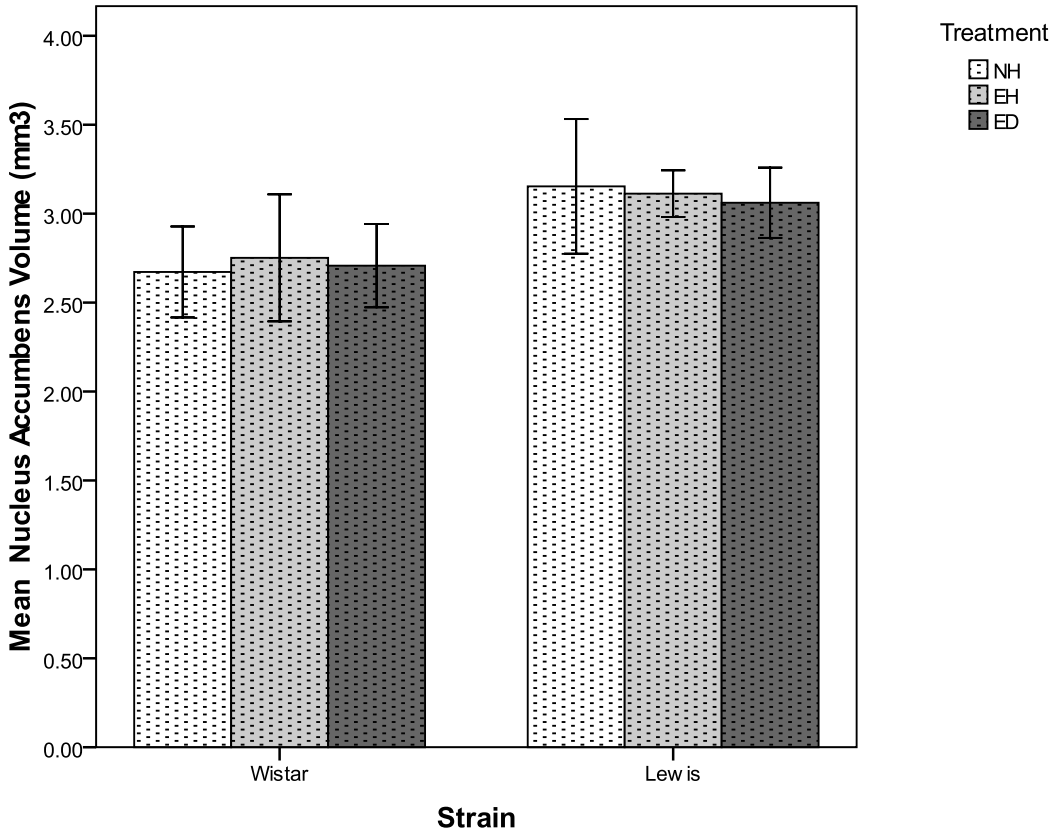

Figure 3.4 - Effects of early deprivation on the nucleus accumbens volume in adult Wistar and Lewis rats.

Data presented as mean ±SEM, n=6 per NH, EH and ED in Wistar's and n=3 NH, n=5 EH and n=6 ED in Lewis rats.

3.2.4 Hematoxylin stained total cell numbers

There was no significant effect of Group on the total number of hematoxylin stained cells any sub-region of the dorsal hippocampal sub regions - CA1 ($F(2, 15) = 0.119$, $p = 0.889$), CA2 ($F(2, 15) = 0.130$, $p = 0.879$), CA3 ($F(2, 15) = 0.523$, $p = 0.603$) and DG ($F(2, 15) = 0.789$, $p = 0.472$) (Figure 3.5).

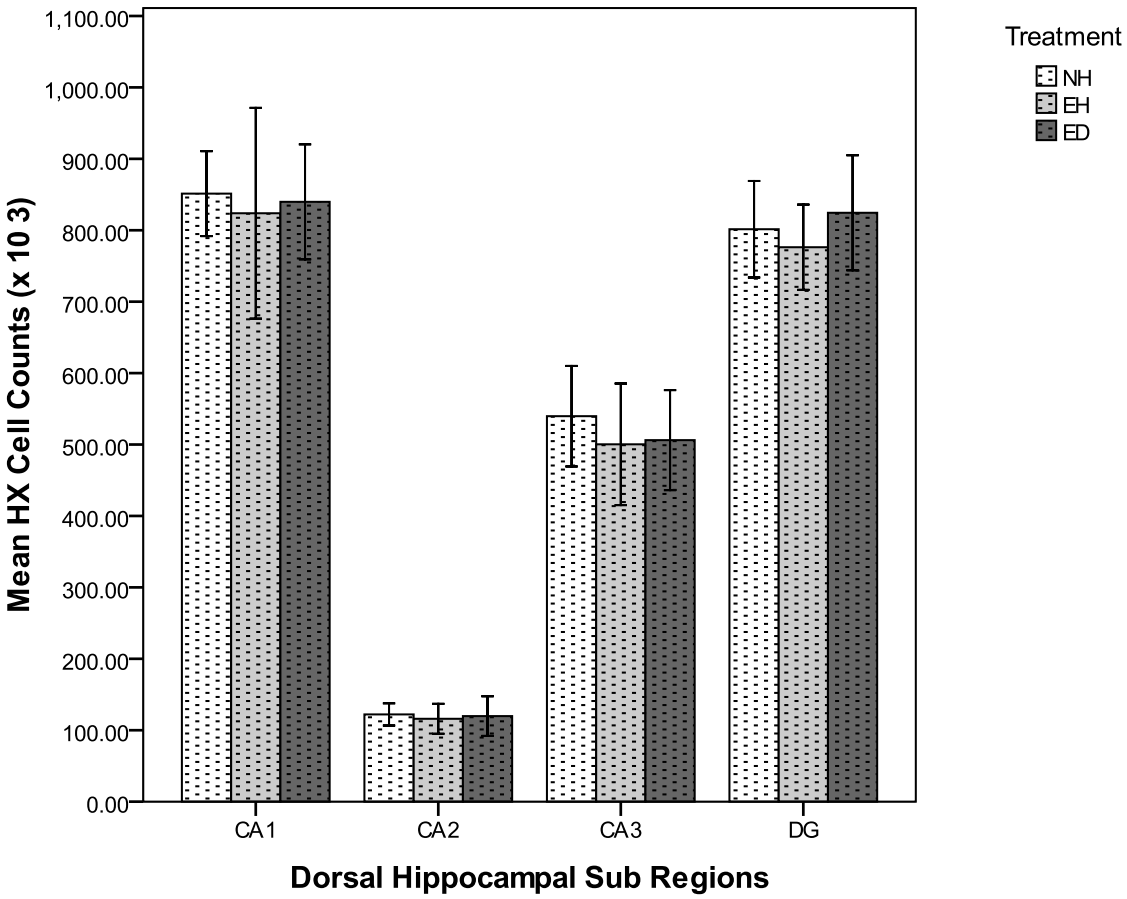

Figure 3.5 - Numbers of hematoxylin positive cells in the dorsal hippocampal sub-regions of non-handled (NH), early handled (EH) and early deprived (ED) adult Wistar rats. Data presented as mean ±SEM, n=6 per group.

Hematoxylin stained nuclei numbers estimated per hippocampal sub-region ($x10^3$)				
	NH	EH	ED	
CA1	851.3 ± 23.1	823.8 ± 57.4	839.8 ± 31.3	
CA2	122.1 ± 6	116 ± 8.2	119.8 ± 10.8	
CA3	539.7 ± 27.4	500.3 ± 33.1	506.5 ± 27.2	
DG	801.4 ± 26.3	776.3 ± 23.2	824.5 ± 31.3	
GFAP +ve cell numbers estimated per region ($X10^3$)				
CA1	120.3 ± 3.1	112.8 ± 4	110.8 ± 3.2	
CA2	17.7 ± 0.8	16.6 ± 0.9	15.8 ± 0.5	
CA3	70.7 ± 2.6	66 ± 1.8	54.3 ± 3.1	**# *Δ
DG	108.5 ± 4	102.8 ± 3.3	97.8 ± 3.4	

Table 3.3 - Estimate of the total hematoxylin stained cell numbers and GFAP-positive cell numbers per dorsal hippocampal sub-region in Wistar rats.

Data are expressed as mean values ± SEM, n=6 per group

*p<0.05, **p<0.01, # vs NH, Δ vs EH

3.2.5 GFAP positive astroglial cell number

In dorsal hippocampus, there was a significant effect of Group on the total number of GFAP positive astroglial cells in Wistar's in CA3 ($F(2, 15) = 11.093$, $p < 0.001$) the number was reduced by an average of 23% in ED vs NH rats ($p<0.01$) and 18% in ED vs EH rats ($p<0.05$). There was no significant effect of Group on this measure in CA1, CA2 and DG (Figure 3.6, Table 3.3).

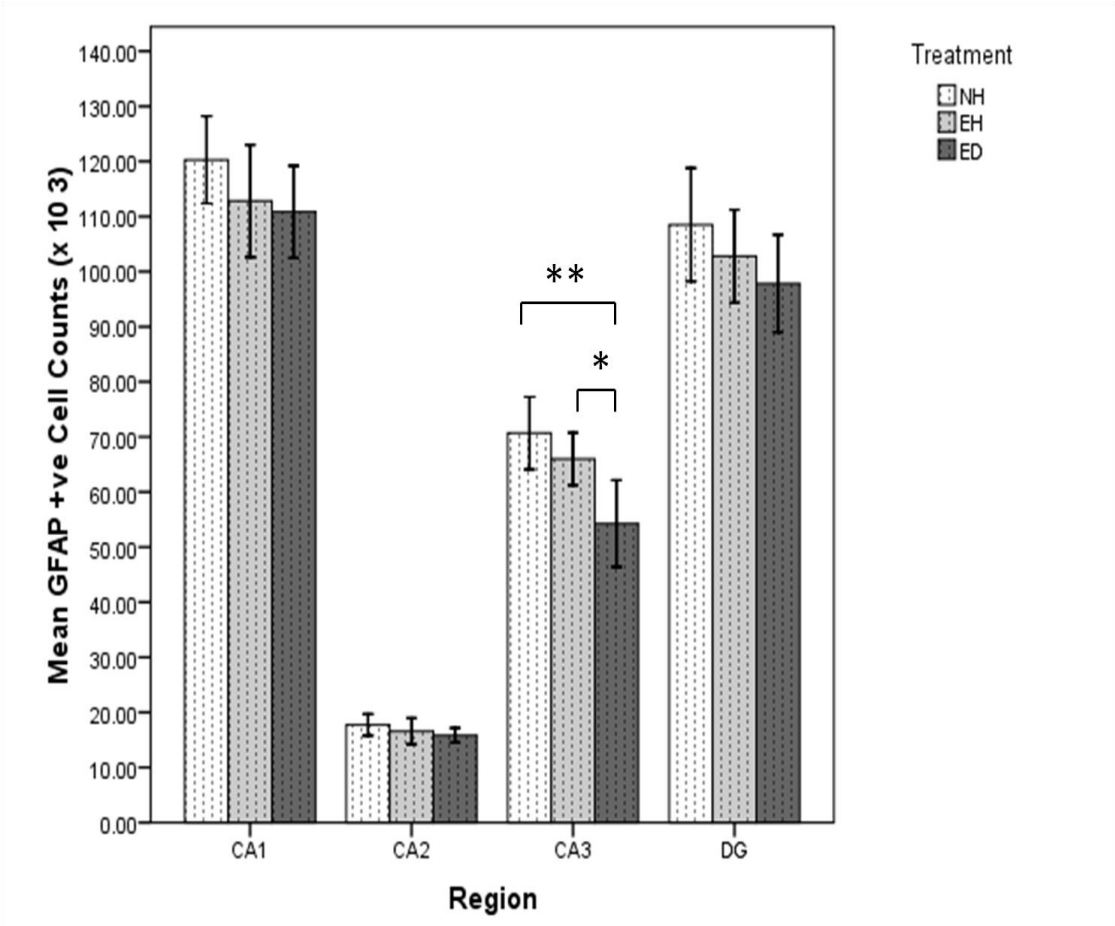

Figure 3.6 - Numbers of GFAP positive cells in the dorsal hippocampal sub-regions of non-handled (NH), early handled (EH) and early deprived (ED) adult Wistar rats.

Data presented as mean ±SEM, n=6 per group, *p<0.05, **p<0.01

3.2.6 GFAP positive astroglial cell morphology

Morphometric data for GFAP-positive astroglia are presented in Table 3.4. There was a significant effect of Group on the total length of primary processes in hippocampal sub-regions: this parameter was decreased in ED vs NH in CA1 (32%, $p < 0.001$), CA2 (19%, $p < 0.01$), CA3 (35%, $p < 0.001$) and DG (23%, $p < 0.01$), and in ED vs EH in CA1 (23%, $p < 0.01$), CA2 (7%, $p < 0.01$) and CA3 (25%, $p < 0.01$).(Figure 3.7) Significant effect of Group on the mean length of primary processes in hippocampal sub-regions: this parameter was decreased in ED vs NH in CA1 (30%, $p < 0.001$), CA2 (20%, $p < 0.05$), CA3 (32%, $p < 0.001$) and DG (21%, $p < 0.01$), and in ED vs EH in CA1 (23%, $p < 0.01$) and CA3 (21%, $p < 0.01$) (Figure 3.8). There was no significant effect of Group on the number of primary processes for any hippocampal sub-region (Figure 3.9).

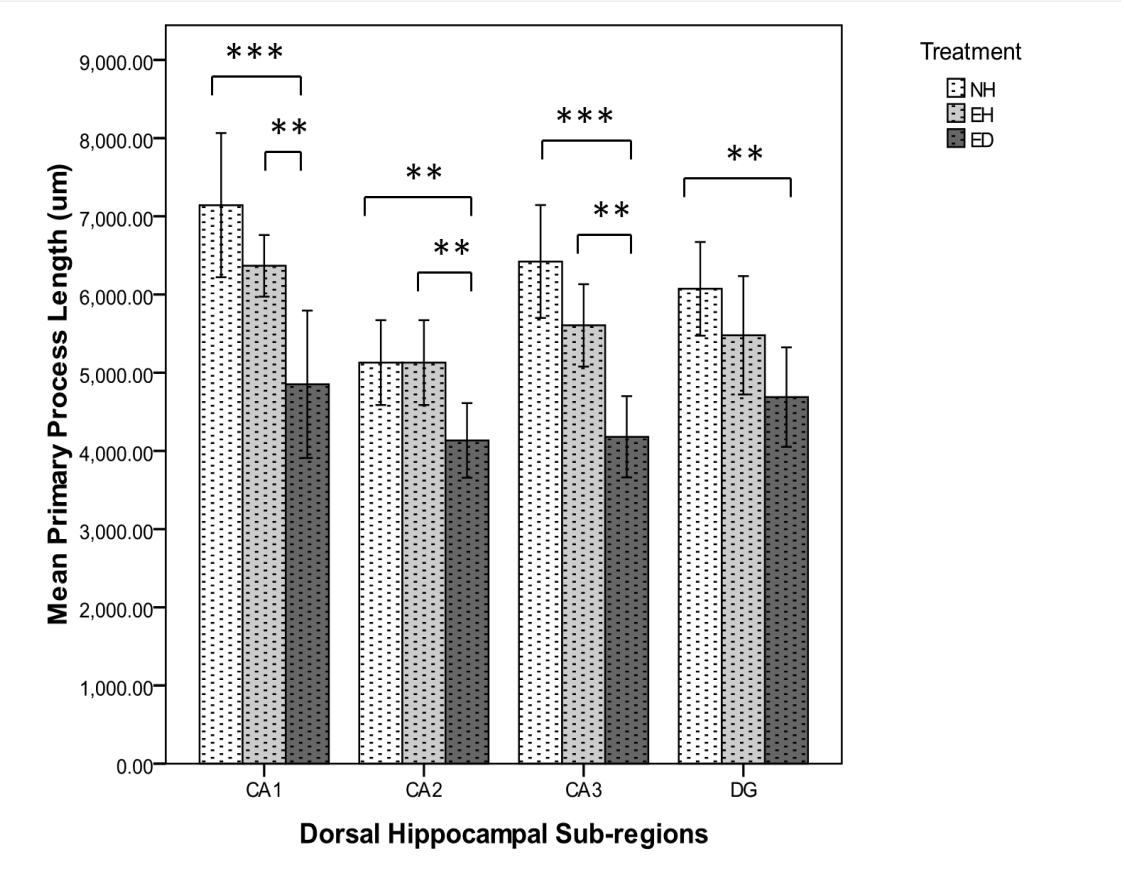

Figure 3.7 - Total primary process lengths of GFAP positive cells in the dorsal hippocampal sub-regions of non-handled (NH), early handled (EH) and early deprived (ED) adult Wistar rats.

Data presented as mean ±SEM, n=6 per group, **p<0.01, ***p<0.001

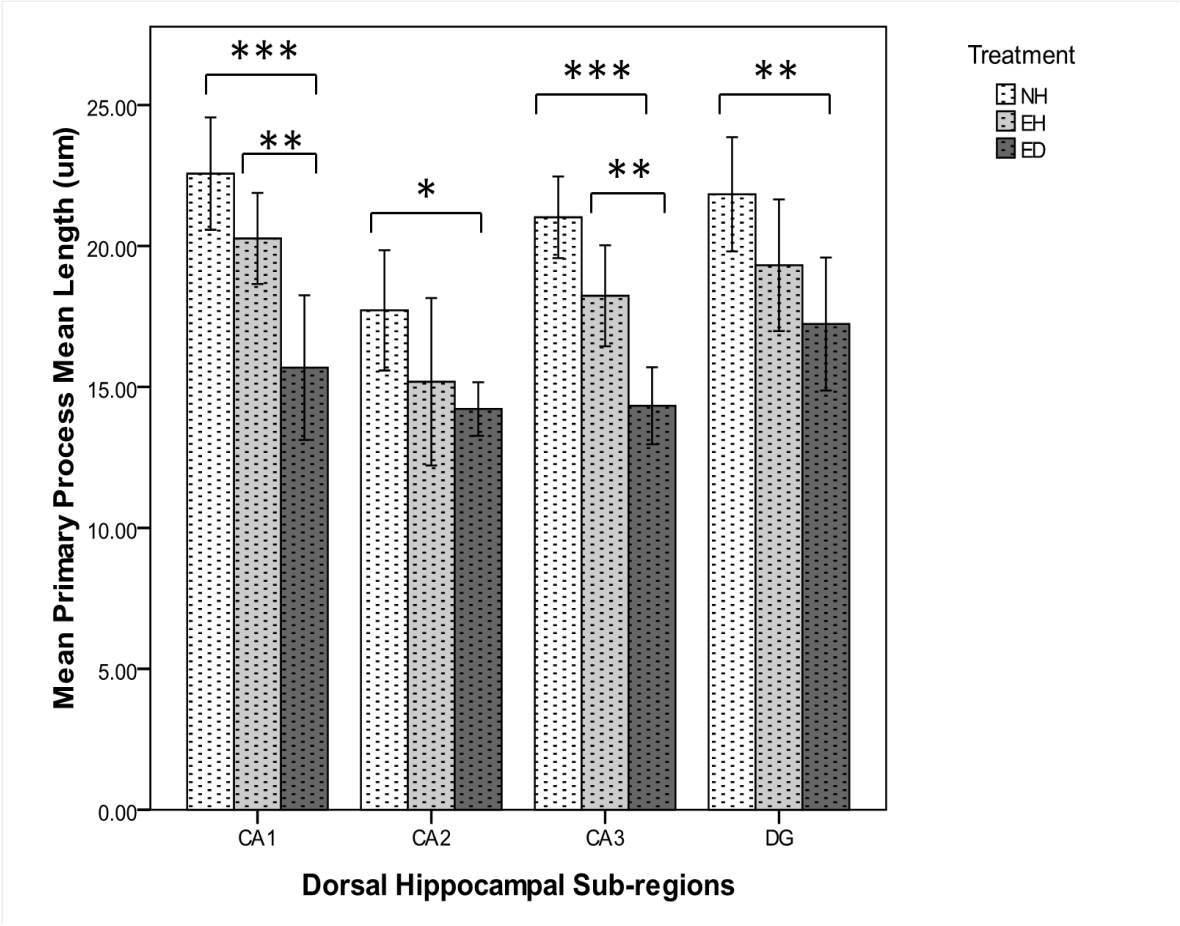

Figure 3.8 - Mean primary process lengths of GFAP positive cells in the dorsal hippocampal sub-regions of non-handled (NH), early handled (EH) and early deprived (ED) adult Wistar rats.

Data presented as mean ±SEM, n=6 per group, *p<0.05, **p<0.01, ***p<0.001

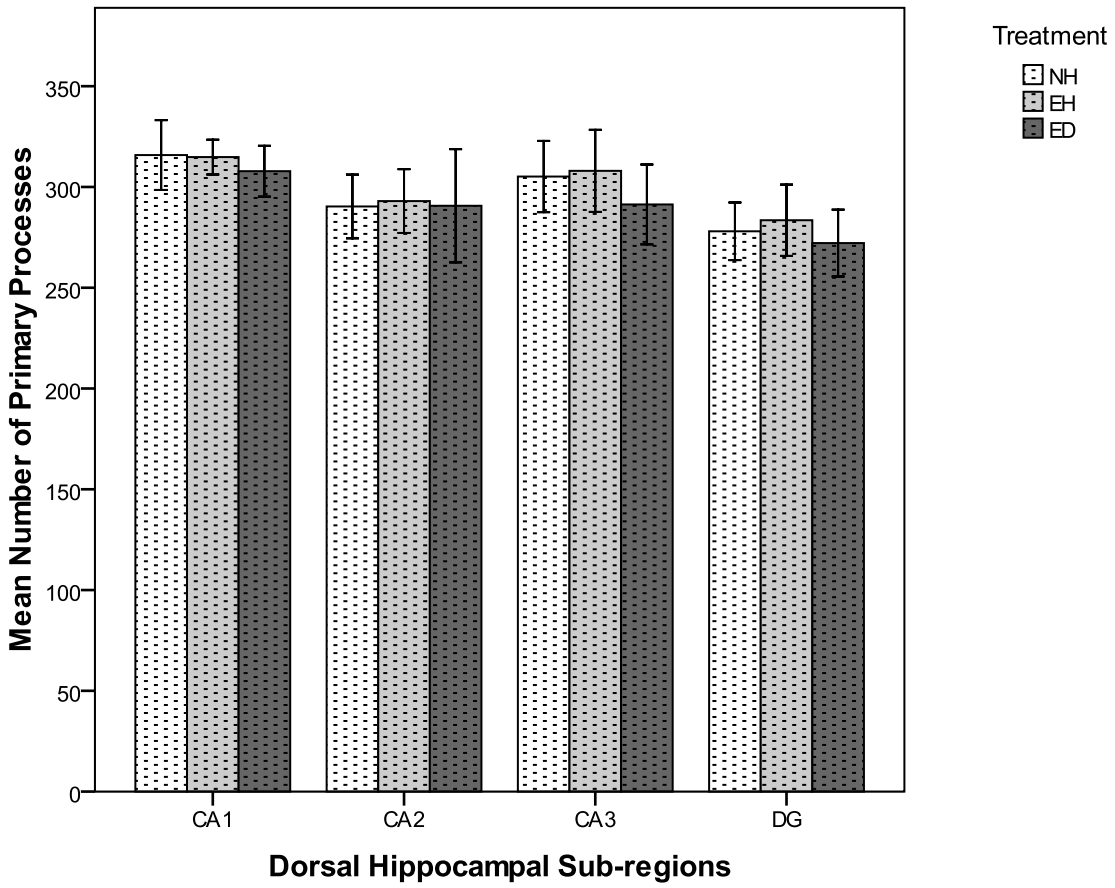

Figure 3.9 - Number of primary processes of GFAP positive cells in the dorsal hippocampal sub-regions of non-handled (NH), early handled (EH) and early deprived (ED) adult Wistar rats.

Data presented as mean ±SEM, n=6 per group

GFAP - astroglia morphology				
Regions	NH	EH	ED	
Total primary process length (µm)				
CA1	7142.8 ± 358.8	6367 ± 153.2	4852.3 ± 366.7	***# **Δ
CA2	5130.0 ± 211.9	4440.8 ± 315.7	4132.9 ± 185.7	**# Δ
CA3	6422.1 ± 280.7	5605.8 ± 204.9	4180.2 ± 202.0	***# **Δ
DG	6073.3 ± 233	5479.1 ± 294.2	4687.4 ± 247.4	**#
Primary process mean length (µm)				
CA1	22.6 ± 0.8	20.3 ± 0.6	15.68 ± 1	***# **Δ
CA2	17.7 ± 0.8	15.2 ± 1.1	14.2 ± 0.4	*#
CA3	21.0 ± 0.6	18.2 ± 0.7	14.3 ± 0.5	***# **Δ
DG	21.8 ± 0.8	19.3 ± 0.9	17.2 ± 0.9	**#
Number of primary process				
CA1	315.8 ± 6.7	314.8 ± 3.4	307.8 ± 4.9	
CA2	290 ± 6.2	293 ± 6.1	290.7 ± 10.9	
CA3	305.2 ± 6.9	308 ± 7.9	291.3 ± 7.7	
DG	278 ± 5.6	283.5 ± 6.9	272.2 ± 6.5	

Table 3.4 - Astroglia (GFAP positive) cell primary process total and mean length and number of primary processes in Wistar rats.

Data are expressed as mean values ± SEM, n=6 per group

*p<0.05, **p<0.01, ***p<0.001; # vs NH, Δ vs EH

3.3 Discussion

The present study based on the stereological estimation found significant dorsal hippocampal volume reductions in the male adult Wistar rats which had experienced early deprivation (12.5% ↓) and early handling (7.5% ↓) with no significant treatment effects in Lewis rats.

The study aimed to assess the early life stress effects on brain remodelling using the volume of specific brain regions involved in stress and depression as the dependent variable. The implication of astroglia was also studied by morphological and cell count analyses. Two rat strains of different responsiveness to stress - Wistar and Lewis were compared. Wistar rats have been found to respond to ED - behavioural and receptor changes have been observed (Leventopoulos et al., 2009). Lewis rats seem to show no negative behavioural response to ED (communication with Dr. Pryce; Kosten and Ambrosio, 2002).

Until now only a few studies in early life stress have used stereological quantitative approach, which allows for unbiased quantitative estimation of brain volume and cell morphology (Schmitz and Hof, 2005). The use of peripheral quantitative computed tomography to estimate the whole brain volume was also a novel attempt towards refinement of volumetric regional analysis, as discussed below. Limbic brain regions which respond to stress such as the hippocampus and amygdala were assessed in order to understand the underpinning neurobiological mechanisms. Brain regions such as dorsal hippocampus, basolateral amygdala and nucleus accumbens which are closely linked to the behavioural symptoms of depressive disorders were the regions of interest.

The present study critically considered the fact that changes in the size of whole brain should be measured and specific brain regions should be scaled to individual brain size, which itself can be a dependent variable. However, assessing brain size is not straightforward and brain weight rather than volume is normally used as the scalar whilst brain regions are usually quantified as volumes. In order to develop a volume-based brain size variable for scaling brain region volumes, we quantified the rat brain volume using the peripheral quantitative computed tomography (pQCT) technique (Shende et al., FENS 2010).

The whole brain volume measurement using pQCT was validated to check its accuracy and reproducibility. The present technique showed a strong correlation between the total brain volume and its weight, and a high precision of the method developed. To our knowledge, this is the first application of pQCT to rat brain volume analysis as part of regional volumetry. With this rigorous methodological approach, no significant treatment effects on the total brain volume were observed.

Behavioural and experimental studies suggest rat strain differences in the response to stress. Lewis rats are relatively nonresponsive to stress, as indicated by reduced synthesis and secretion of CRH and are known for their decreased responsiveness to the negative feedback loop of ACTH and CRH in the HPA axis (Grota et al, 1997). Differences in mineralocorticoid receptor messenger RNA expression, receptor numbers, receptor affinity, or receptor activation has not been detected between the two strains (Oitzel et al, 1995), suggesting that possible difference may lie in the transduction of negative feedback signals. It has been suggested that differences in neuroendocrine and/or neurochemical (more specifically dopaminergic) state may be responsible for the differential response to early manipulation (Varty and Geyer

1998; Kinney et al. 1999). It is of interest to note that maternal deprivation in Wistar rats leads to an increase in mRNA expression for tyrosine hydroxylase within the substantia nigra pars compacta resulting in hyper-responsiveness to stress (Rots et al. 1996). It has also been suggested that the reduced level of corticosterone released by the Lewis rat is insufficient to terminate inflammatory responses, and as a result, they show an increased susceptibility to autoimmunity (Sternberg et al, 1992).

The present data provide support for the theory that genetic and environmental factors may contribute to the overall development of the brain and brain function (Kinney *et al*, 1999), and suggest that early life deprivation may represent an interesting tool for studying the interaction between genetic and early stressful life events.

The extent of the observed hippocampal volume reduction is similar to the studies reported in the clinical (Viderbech and Ravnkilde, 2004) and experimental studies (Czeh et al., 2010). Various in vivo imaging studies of patients with major depressive disorder have shown selective functional and structural changes in the hippocampus (Sheline *et al*, 1996). Another in vivo MRI study documented significant reduction (10 -15%) in hippocampal volume supporting the present study (Cambell *et al*, 2006; Videbech & Ravnkilde, 2004). A stereology study carried out on maternally deprived Wistar rats reported similar decrease in hippocampal volume with no evidence of cell loss and significant reduction in astrocytic process length (Opacka-Juffry *et al*, 2008).

It should be noted that the change observed in the present study appear to be region-selective as neither amygdala nor nucleus accumbens show treatment-dependent

volume changes. Also, only few stereological analyses have been performed and currently unbiased stereological cell counting for volumetric and morphological techniques are the method of choice for evaluating changes in specific brain regions.

There are various mechanisms which could underpin the observed hippocampal shrinkage in early deprived and early handled rats, such as a stress-induced reduction in neurogenesis, alterations in gliogenesis, glial numbers and morphology, apoptosis or cell death, extracellular space and fluid content (review by Czéh and Lucassen, 2007). The present study suggests the implication of astroglial density and morphology in the hippocampal volume loss. It is also plausible that these could be the reasons for volumetric changes reported in patients suffering from stress-related depressive disorders.

The amygdala responds to fearful stimuli (Whalen *et al*, 1998) whereas nucleus accumbens most consistently respond to rewards (Ernst *et al*, 2005). Studies have shown ED in rats leads to anhedonia indicating brain reward system to be likely implicated (Leventopoulos et al., 2009). Together the amygdala and nucleus accumbens mediate detection and reaction to motivating stimuli.

To date there are a number of neuroimaging studies carried out in patients suffering from depression which suggests volumetric changes in the limbic system. Patients with major depressive disorder have shown disturbed activation in the limbic brain structures (Monk *et al*, 2008).

Studies have suggested role of MePD and MeA regions of amygdala in several sexually dimorphic social behaviors in the adult animal has been observed with increase in their regional volume and neuronal soma size (Morris *et al*., 2005). Also

structural magnetic resonance imaging (MRI) studies indicates an abnormal growth pattern in the amygdala marked by precocious enlargement in autism patients (Sparks *et al.*, 2002; Schumann *et al.*, 2004). But contradictory results have been found in amygdala volumes in major depression. A study carried out by Bremner *et al* (2000) identified an increase in right amygdala volume. A study carried out by Yossuck *et al* (2006) showed no significant differences in the basolateral amygdala volume in rats exposed to corticosteroid. However, study carried out by Sheline *et al* (1998) in MDD patients have found a reduction in the amygdala core nucleus volume.

Very few post-mortem experimental animal studies have been performed to confirm the volumetric changes in amygdala and nucleus accumbens. Also less focus has been on basolateral amygdala which is involved in the emotional memory formation and its link with hippocampus related memory formation. The amygdala and nucleus accumbens are difficult structures to measure, since in many areas the cortical amygdala merges with surrounding cortex, and specific boundaries selected varied greatly in different studies and also it is difficult to differentiate between the nucleus accumbens boundary and its surrounding areas.

No significant differences were found in both basolateral amygdala and nucleus accumbens volume in both Wistar and Lewis rats, thus indicating that the hippocampus is the most vulnerable region of early life stress.

Animal behavioural studies have shown involvement of the amygdala and accumbens in the emotional regulation and early life stress. In the present study no volumetric changes were seen in basolateral amygdala and nucleus accumbens

suggesting there may be other mechanisms involved which disrupts their functioning like receptor changes and/or implications of neuronal and glial cells.

The present study also focussed on the hippocampal astroglia considering their major role in maintaining brain plasticity at the level of synapses and cells (Theodosis et al., 2008) and the evidence that astrocytes are reduced in depression (Harrison, 2002). In terms of methodology, GFAP immunolabelling of astroglia which was used in the present study occasionally attracts criticism that it does not label all astroglial cells in the brain (Reichenbach and Wolburg, 2005). However, a study compared S100 and GFAP (both are selective marker for astroglial cells) showed that both markers stain almost 80% the hippocampal astroglial cells (Ogata and Kosaka, 2002; Wei et al., 2009). Accepting the limitations of GFAP labelling, the present study aimed at estimating the length and numbers of primary astrocytic branches where GFAP is reliably detectable (Theodosis et al., 2008).

Both early deprivation and early handling compared to non handling led to significant reduction only in the hippocampal CA3 – GFAP positive cell numbers in Wistar rats. No changes in the total hematoxylin stained cell count were observed across all experimental groups. Similar changes in the astroglial cell loss were reported in female rats exposed to prenatal stress (Behan et al., 2011) and in WKY rats exposed to stress (Gosselin et al, 2009). Rajkowska and Miguel-Hidalgo (2007) postulate that decreased density of astroglia and reduced GFAP expression are associated with early onset depression.

The current study also found a significant decrease in the length of the total and mean primary processes, with no changes in the number of primary processes in the hippocampal CA1, CA2, CA3 and DG subregions in ED relative to NH and EH rats.

The present observed astroglial morphological abnormalities of hippocampus which are likely to have functional implications, as a reduction in the length of primary processes would affect glia-occupied space and glia-neurone interactions which are essential for hippocampal activity (Theodosis et al., 2008). Interestingly, an increase in astrocytic primary process length in response to enriched environment was recently reported in male mice (Viola et al., 2009).

On the basis of the present findings, the ED effects on astroglia can be interpreted as a manifestation of long-term reductions in brain plasticity. As such the present study findings support the growing consensus that it is astrocytic-neuronal plasticity which is a major dynamic feature of the brain when responding to positive or negative environmental events (Theodosis et al., 2008).

The observed impoverishment of astroglial morphology in the present study can be treated as a demonstration of glial plasticity, a phenomenon that deserves more research in the context of the effects of stress-related pathologies.

3.4 Conclusions

The present study demonstrates long-term effects of early life stress on hippocampal remodelling with the contribution of astroglia; this effect appears to be rat strain-dependent. In terms of methodology, peripheral quantitative computed tomography was employed as a novel approach to measure total brain volume post-mortem in a high-precision manner, which is important as overall brain size itself is a variable in neuroanatomy and neurobiology research. In this respect, the present study shows that pQCT can be used as a tool for soft tissue (brain) and not only hard (bone) tissue volume analysis. This approach is of relevance to volumetric studies on neuroplasticity where regional volumes need to be scaled to the total brain volume.

The current finding of hippocampal volume shrinkage is of relevance to elucidating the significance of decreased hippocampal volume to the neuropathology of depression.

Stereological estimation of the basolateral amygdala and nucleus accumbens volume show no significant effects, which suggests an involvement of a different mechanism which disrupts emotional regulation and motivation stimuli towards reward observed in vivo.

The study demonstrates that early life stress in the form of repeated disruption of mother - infant interactions leads to long-term effects on the dorsal hippocampus in terms of reduced volume, impoverished astroglial morphology in subregions CA1, CA2, CA3 and DG, and reduced numbers of GFAP-positive astroglial cells in CA3. Therefore, the present study provides evidence for long-term remodelling of hippocampal volume and astroglia following early life stress in Wistar rats.

Chapter 4

Prenatal exposure to glucocorticoids: effects of dexamethasone treatment on brain volume and astroglia

4.1 Introduction

As overviewed in Chapter 1, the prenatal period of life is the most critical and sensitive phase where non-genetic/programming factors could mark permanent alterations in behaviour and physiological functions (Barker, 1995; Seckl, 2001). The environmental challenges affecting foetal development of the pregnant female are due to stress, which increases the HPA axis activity leading to increased plasma glucocorticoid (GC) – the natural GC receptor (GR) ligand (Seckl, 2004). However, synthetic GC, such as dexamethasone (Dex), also act through specific binding to the GR and are one of the programming factors during prenatal/developmental plasticity (Welberg et al., 2001). Dex is also known to modulate the expression of the genes that contain GC responsive elements in their promoter (De Kloet et al., 1998). Therefore, both prenatal stress and prenatal GC exposure result in GR activation in the foetus.

The foetal exposure to synthetic corticosteroids during the prenatal period affects the normal brain development, causing long term changes in the brain structure and function with cognitive, behavioural and emotional disturbances (Matthews, 2000; Yossuck et al., 2006). Prenatal corticosteroid treatment started in 1972 (Liggins & Howie, 1972) having been considered as safe and without side effects (NIH consensus, 1994; 1995) and used clinically (Matthews, 2000). In the 1990's, 98% of the British obstetric clinicians prescribed repeated courses of the prenatal corticoid therapy (Brocklehurst et al., 1999). About 85% of the neonates with prenatal corticosteroid therapy received multiple courses (Empana et al., 2004).

The proportion of preterm births is considerable, for example in England and Wales in 2005 alone over 11,500 infants (8%) were born prematurely at less than 33 weeks of gestation (Moser et al., 2007) and dexamethasone is commonly administered to ventilator-dependent premature infants with chronic lung insufficiency to improve lung function (Romagnoli et al., 2002; Charil et al., 2010) and normal foetal development (Ward, 1994).

Neurodevelopmental effects of prenatal dexamethasone treatment need more attention as there are associations between GC and risk of neuropsychiatric disorders including depression and schizophrenia (Koenig et al., 2002; Huttunen et al., 1994; Yu et al., 2008; Steckler et al., 1999). Neurodevelopmental delay and cerebral palsy along with differences in morphology and structure of the offspring's brain treated with Dex have been reported in humans (Shinwell et al., 2000; Halliday, 2002; Murphy et al., 2001; Choi et al., 2004). Corticosteroids are also involved in the regulation of prostaglandin synthesis, gluconeogenesis in liver cells and modulation of immune response (Wiegers and Reul, 1998). In rats, both prenatal stress and prenatal GC exposure have been associated with increased anxiety (Estanislau and Morato, 2006; Welberg et al., 2001), impaired HPA negative feedback (Weinstock, 2005; Welberg et al., 2001) and impaired memory (Brabham et al., 2000; Zagron and Weinstock, 2006) in adulthood. Prenatal stress has been also linked with increased behavioural despair (Smith et al., 2004) and impaired social behaviour (Patin et al., 2005).

It is plausible to assume that the behavioural effects couple with anatomical changes in the brain as preclinical studies have demonstrated that dexamethasone plays a role

in brain development by terminal maturation, remodelling of axons and dendrites and cell survival (e.g. Yehuda et al., 1989). Indeed, premature infants treated with dexamethasone postnatally had cortical brain volume reduced by 35% when compared to full term controls (Murphy et al., 2001). Previous studies have also suggested effects of corticosteroids on the HPA axis, which lead to changes in their negative feedback and result in hippocampal damage with cell and volume loss (Uno et al., 1990; 1994). However, the neurobiological mechanisms of perinatal corticosteroid treatment remain unclear.

During the prenatal period (lasting from fertilisation to birth), the HPA axis components start to emerge and function (Challis et al., 2001). In rodents, it is gestational day 18 when the foetal HPA axis is responsive (Ohkawa et al., 1991). The foetal HPA axis can be activated by maternal CRH via placenta which makes the prenatal period complex and interesting where the stress system of the mother can influence their offsprings stress system (Weinstock, 2005). The foetal exposure to corticosteroid associates a link between prenatal environment and disorders with HPA axis dysfunctioning (Seckl, 2004). Prenatal corticosteroid exposure causes elevated levels of basal and stress induced plasma corticosterone in adult rats (Levitt et al., 1996) which leads to attenuation of the HPA axis feedback mechanism (Seckl, 2004). Reduction in the hippocampal glucocorticoid and mineralocorticoid receptor density may result in HPA axis dysfunction (Welberg et al., 2001). Moreover, development of the adult hyperglycaemia and hypertension along with behavioural changes and HPA axis activation are associated with the prenatal dexamethasone exposure (Lewitt et al., 1996; Welberg et al., 2001). The sensitivity of the developing hippocampus is of particular concern, because it becomes an important brain center

after birth, involved in the regulation of the hypothalamic–pituitary–adrenal (HPA) axis.

Postnatal corticosteroid effects have been well researched (McEwen., 1994; Sapolsky., 1996; Joels., 2001) but less attention has been paid to prenatal corticosteroid treatment effects (Matthews., 2000). As prenatal corticosteroid administration is the practised treatment to improve lung function it is important to understand its chronic effects.

Two possibilities of foetal programming - foetal malnutrition or foetal over exposure to glucocorticoids, have direct or indirect effects on foetal development (Barker et al., 1993a; Matthews, 2000). Exposure to the synthetic glucocorticoid like Dex may affect the specific foetal brain structure and/or general cell processes related to CNS accounting for long term effects. Although Dex is very potent, especially because it is not readily metabolised by the foetus, the pharmacological studies support the view that the placental transfer of maternal cortisol is a primary mediator of maternal stress effects on foetal development (Dodic et al., 2002; Kay et al., 2000; Pepe and Albrecht, 1987).

As mentioned earlier, the direct effects of glucocorticoids on brain development are mediated through corticosteroid receptors: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Prenatal glucocorticoid treatment is often GR specific where dexamethasone, binds exclusively to GR (Reul and de Kloet, 1985). The GR is identified throughout the brain; however both GR and MR are highly expressed in hippocampus. During the foetal and postnatal life these receptors follow a unique pattern of expression especially in the hippocampus (Rosenfield et al.,

1988; Vazquez et al., 1996). Studies have shown that disruption in the maternal care causes alterations in the GR development causing altered hippocampal function (Francis et al., 1999; Liu et al., 1997).

Prenatal corticosteroids such as betamethasone (5–16 doses, 12 mg each, administered twice weekly) have shown short term effects like transient hypertrophic cardiomyopathy in newborns (Yunis et al., 1999). Long term effects such as increased mortality, decreased foetal growth and birth weight, adrenal suppression and reduced head circumference were observed with multiple betamethasone prenatal administration (French et al., 1999; Banks et al., 1999). Long term prenatal stress, indicated with prolonged increased maternal corticosteroid levels, was associated with increased neurological dysfunction, developmental delay (late or poor walking, speech deficits), and behavioral disturbances (such as restlessness and poor interpersonal skill development) in children (Stott., 1973). In rodents, prenatal corticosteroid exposure has multiple effects on the hippocampal cell proliferation, neurotransmitter turnover and receptor expression (DeKloet et al., 1998; Seckl., 1998). Prenatal dexamethasone causes decrease in body and brain weight in rats (Carlos et al., 1992), decrease in postnatal protein/DNA ratio (Slotkin et al., 1992) and decrease in both GR and MR in the hippocampus (Welberg et al., 2001).

Studies designed to assess perinatal dexamethasone exposure outcomes have suggested a close association between environmental challenge during pregnancy, altered foetal growth, development and later pathophysiology (Seckl, 1998) along with its involvement in mediating the detrimental effects of stress (Coe & Lubach,

2005). Increased prevalence of schizophrenia has been observed in children subject to prenatal maternal stress (Huttunen et al., 1994; Van Os & Selten, 1998) suggesting perinatal environment as critical phase for plasticity.

Human infants treated with prenatal dexamethasone administration had lower birth weight (Stark et al., 2001), smaller head circumference, lower IQ scores, poor motor and visual - coordination skills during their school age (Yeh et al., 2004). Premature infants treated with Dex often suffer from affective and cognitive disorders in adulthood such as autism, attention deficit hyperactivity disorder, learning difficulties and depression (Thompson et al., 2001; O'Donnell et al., 2009). Interestingly, prenatal exposure to the stress of a moderately severe natural disaster (ice storm crisis in the Canadian province of Québec) is also associated with lower cognitive and language abilities (Laplante et al., 2008). In rats, spatial learning impairment is one of the consistent findings in the offspring of mothers treated with dexamethasone prenatally in drinking water (Brabham et al., 2000). High anxiety behaviour (Vallee et al., 1997), changes in spontaneous motor activity (Diaz et al., 1997), altered performance in the open field and forced swim test (Welberg et al., 2001) are other observations in rats exposed to prenatal dexamethasone.

As mentoned in the general introduction, the brain limbic system plays an important role in the regulation of emotion, behaviour and memory processes (Sapolsky, 2000). During the prenatal brain developmental period, the limbic regions, such as the hippocampus and amygdala, are sensitive to both endogenous and exogenous corticosteroid (Matthews, 2000; Halasz et al., 2002). Exposure to corticosteroids during the critical period of foetal development affects the limbic structures resulting

in cognitive and neuroendocrine dysfunction (Matthews, 2000). Several lines of evidence indicate an alteration in the cytoarchitecture of hippocampus, a region with high glucocorticoid receptor density, as a result of prenatal stress. Spatial learning impairment (Lemaire et al., 2000), long term potentiation reduction (Son et al., 2006) and decline in cognitive processes (Hayashi et al., 1998) correlates with hippocampal dysfunctioning observed in the rats exposed to prenatal stress. The prevalence of cognitive impairments was higher in males than in females following dexamethasone administration (Brabham et al., 2000).

Magnetic resonance imaging (MRI) studies in the prematurely born children treated with dexamethasone have confirmed brain structural abnormalities (Murphy et al., 2001). The cortical brain volume reduction due to postnatal corticosteroid treatment is one of the published examples (Murphy et al., 2001). Reductions in the regional volume of the hippocampus, amygdala and corpus callosum were reported in the prematurely born children (Abernethy et al., 2002; Peterson et al., 2000; Nosarti et al., 2002). However, the mechanisms underlying these brain volume changes in association with the corticosteroid treatment used for prevention of chronic lung disease in premature babies are not fully understood (Tijsseling et al., 2012). Hippocampal atrophy and volume loss have been revealed by means of the MRI technique in the children who survived from very low birth weight (Abernethy et al., 2002) and also in monkeys and rats treated with high GC doses (Woolley et al., 1990; Sapolsky et al., 1990). Prenatal exposure to DEX has been shown to cause degeneration of hippocampal neurones and reduce hippocampal volume in adolescent rhesus macaques (Uno et al., 1990). Coe et al. (2003) also observed volume loss by 10-12% in the offspring of stressed pregnant rhesus monkeys.

Degeneration of hippocampal CA1 and CA3 cells and dendritic branches under dexamethasone exposure have been reported in preclinical rodents (Haynes et al., 2001; Sousa et al., 1999) and primate studies (Sapolsky et al., 1990). Prenatal exposure to dexamethasone has been shown to delay maturation of neurons and inhibit neurogenesis in rats (Lupien et al. 2009). Hippocampal glucocorticoid receptors, brain-derived neurotrophic factor, corticotropin releasing hormone (CRF) and neuropeptide Y, have all been recognised as possible targets of prenatal corticosteroid treatment resulting into reprogramming of these molecules (Velisek., 2005).

In addition, studies have reported delays in the maturation of astrocyte and capillary tight junction, and myelination of the corpus callosum in sheep (Antonow-Schlorke et al., 2009) and a significant decrease in spine density of the hippocampus in rats (Hayashi et al., 1998) following exposure to prenatal synthetic glucocorticoids. Morphological changes in astroglia have also been reported in response to stress. Thus studies have shown long lasting astroglial reactions (increased cell area of GFAP positive cells and high S100 protein levels) and decreased dendritic arborisation in response to prenatal stress (Barros et al., 2006) and increase in the glial fibrillary acidic protein (GFAP) immunoreactivity in the hippocampal CA1 region and striatum following repeated immobilisation stress (Kwon et al., 2008). There is growing appreciation for the importance of astrocytes, non-neuronal glial cells, in the overall brain functioning. Reduction in glial cell numbers has been reported in the post-mortem studies on patients suffering with major depressive disorder (Bowley et al., 2002). Studies have also shown reductions in glial cell proliferation *in vitro* (Crossin et al., 1997) and *in vivo* (Wennstrom et al., 2006) in the

course of corticosteroid exposure. Till date no reports have been published on effects of prenatal GC treatment on astroglial cells although evidence for the role of astroglia in brain plasticity has increased in recent years (Jauregui-Huerta et al., 2010).

4.1.1 Aims and Hypotheses

The present study was designed to investigate the effects of prenatal exposure to dexamethasone on the volume of the hippocampus and the morphology of its astroglia in an attempt to evaluate hippocampal remodelling and the role of astroglia in this process. The specific aims of the present study were to investigate if:

- Prenatal dexamethasone exposure will be associated with a reduction in the volume of hippocampus, amygdala and nucleus accumbens regions.
- Prenatal dexamethasone exposure will not affect the total cell number in hippopcampus, basolateral amygdala and nucleus accumbens regions.
- Prenatal dexamethasone exposure will result in reductions of GFAP positive astroglial cell primary process length.

4.2 Results

4.2.1 Whole brain volume and brain weight

Independent t-test was performed to test the treatment effect between groups (Table 4.1). No significant difference was observed suggesting no treatment effect on whole brain volume (p= 0.376) and brain weight (p = 0.442). The results suggest that the prenatal dexamethasone exposure does not alter the whole brain volume and brain weight.

	Control	Dex Treated
Brain Weight (g)	1.74 ± 0.02	1.77 ± 0.03
Brain Volume (cc)	1650.18 ± 17.75	1681.24 ± 28.43

Table 4.1 - Effects of prenatal dexamethasone exposure on brain weight and volume of male adult Sprague Dawley rats. The brain weight and volume are expressed as mean ± SEM (n = 6 per group).

4.2.2 pQCT analysis

In order to estimate the precision and reproducibility of the technique, repeated pQCT scanning was done 5 times for the same rat brain and whole brain volume was estimated. The data obtained showed 0.26% of coefficient of variation (CV) and -0.0001% of average measurement error.

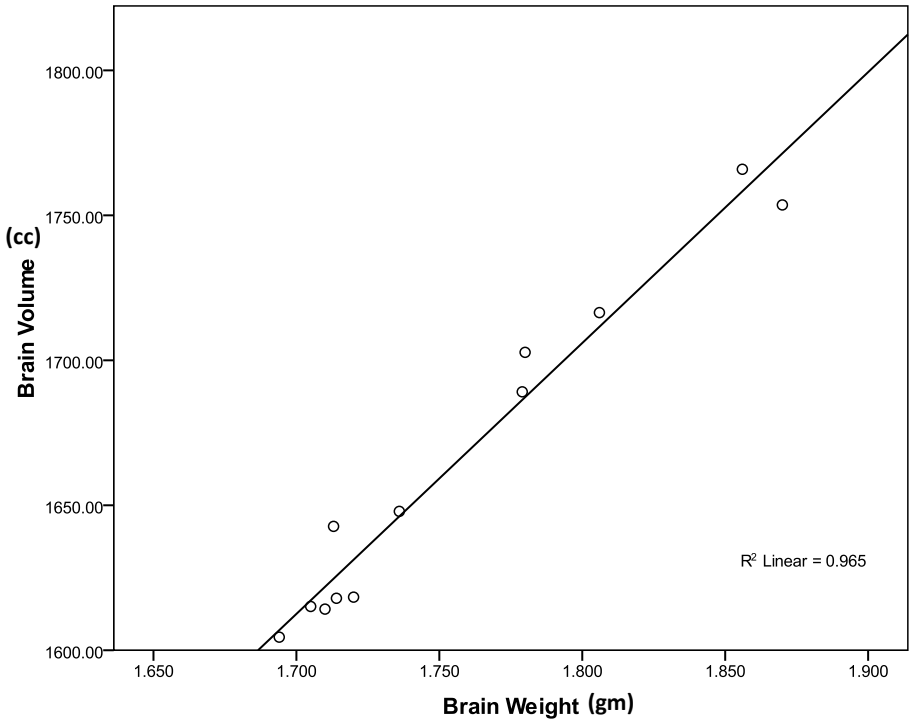

Figure 4.1 - Scatter plot showing the strong positive linear relationship between brain weight and volume (n=12).

The Pearson correlation test was used to identify the relationship between brain weight and volume. A strong positive linear correlation ($r = 0.983$, $p<0.01$) was observed between brain weight and volume. Also, linear regression demonstrated a significant positive relationship ($F (1, 10) = 278.472$, $p<0.001$, $R^2 = 0.965$) (Figure 4.1).

4.2.3 Regional volume analyses

4.2.3.1 Dorsal Hippocampus

The dorsal hippocampus volume was estimated using stereology with Cavalieri principle (StereoInvestigator, MBF). Independent t-test was used for statistical

analysis in order to compare the means representing the control and DEX treated group. DEX treated rats showed a significant decrease in the dorsal hippocampal volume by 6.5% vs control ($p<0.05$). There was a significant reduction by 8% ($p<0.05$) in the relative volume of the dorsal hippocampus expressed as percentage of the whole brain volume when compared with the control (Figure 4.2, Table 4.2).

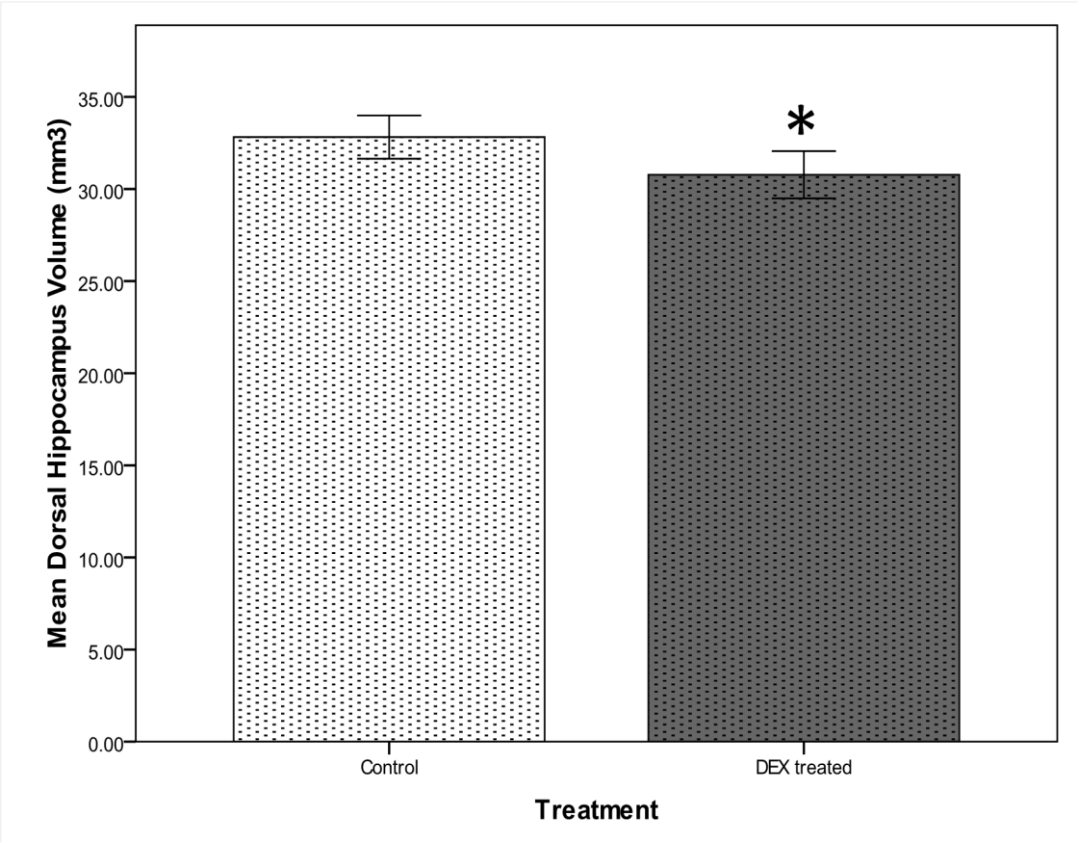

Figure 4.2 Effect of prenatal dexamethasone treatment on the dorsal hippocampal volume in adult male Sprague Dawley rats.

The prenatal dexamethasone exposure resulted in reduction of dorsal hippocampal volume. Independent sample t-test revealed significant differences (*$p<0.05$)

Volume		
	Control	Dex Treated
Absolute values (mm^3)		
Dorsal Hippocampus	32.8 ± 0.46	30.8 ± 0.50 *
Basolateral Amygdala	0.81 ± 0.02	0.80 ± 0.02
Nucleus Accumbens	2.28 ± 0.06	2.26 ± 0.07
Relative values (as % brain volume)		
Dorsal Hippocampus	1.99 ± 0.04	1.83 ± 0.04 *
Basolateral Amygdala	0.046 ± 0.001	0.048 ± 0.001
Nucleus Accumbens	0.138 ± 0.004	0.135 ± 0.005

Table 4.2 – Effects of prenatal dexamethasone exposure on the regional brain volume of male adult Sprague Dawley rats.

Data are expressed as mean ± SEM (n = 6 per group). *p<0.05

4.2.3.2 Basolateral Amygdala and Nucleus Accumbens

The basolateral amygdala and nucleus accumbens volume (right side) were estimated using stereology with Cavalieri principle (StereoInvestigator, MBF). Independent t-test was used for statistical analysis to compare the means between the control and DEX treated groups. No significant differences were observed between the treatment groups in the amygdala (p= 0.834) and nucleus accumbens (p= 0.905) (Table 4.2).

4.2.4 Total and GFAP positive cell numbers

The total number of HX stained cells in the dorsal hippocampal sub regions CA1 (P_{value}= 0.821), CA2 (P_{value}= 0.975), CA3 (P_{value}= 0.991) and DG (P_{value}= 0.959) did not reveal a significant main effect of treatment (independent t-test). The total number of GFAP positive astroglial cells in the dorsal hippocampal sub regions was estimated. There were no significant changes seen in GFAP-positive cell population

in the dorsal hippocampal sub regions (independent t-test) CA1 (p= 0.607), CA2 (p= 0.517), CA3 (p= 0.500) and DG (p= 0.424) in response to the DEX treatment (Table 4.3).

Hematoxylin stained cell numbers estimated per region (x10^3)		
	Control	Dex Treated
CA1	706.8 ± 8.8	714.1 ± 29.7
CA2	95.9 ± 3.4	95.8 ± 2
CA3	491.3 ± 37.2	490.7 ± 32.6
DG	791.4 ± 31.6	789.1 ± 30.6
GFAP +ve cell numbers estimated per region (x10^3)		
CA1	219.4 ± 6	213.6 ± 9.1
CA2	26.8 ± 1.1	25.8 ± 1
CA3	110.9 ± 6.1	104.4 ± 6.9
DG	201 ± 4.7	195.3 ± 5.1

Table 4.3 – Effects of prenatal dexamethasone exposure on the number of total and GFAP positive cells in male adult Sprague Dawley rats.

Data are expressed as mean ± SEM (n = 6 per group).

Hematoxylin stained cell numbers estimated per region (x10^3)		
	Control	Dex Treated
Basolateral amygdala	52.86 ± 3.4	58.79 ± 2.4
Nucleus accumbens	146.58 ± 6.8	139.61 ± 6.4
GFAP +ve cell numbers estimated per region (x10^3)		
Basolateral amygdala	23.68 ± 1.9	25.71 ± 2.1
Nucleus accumbens	20.73 ± 1.8	18.28 ± 2.0

Table 4.4 – Effects of prenatal dexamethasone exposure on the number of total and GFAP positive cells in male adult Sprague Dawley rats in basolateral amygdala and nucleus accumbens region.

Data are expressed as mean ± SEM (n = 6 per group).

The total number of HX stained cells ($p= 0.180$) and GFAP positive cells ($p= 0.495$) in the basolateral amygdala did not reveal a significant main effect of treatment (independent t-test). The total number of HX stained cells ($p= 0.474$) and GFAP positive cells ($p= 0.384$) in the nucleus accumbens did not reveal a significant main effect of treatment (independent t-test) (Table 4.4).

4.2.5 GFAP positive - Astroglia cell morphology

Morphological analysis of the GFAP positive astroglia revealed significant changes in the total primary process length and primary process mean length. Independent t-test analysis showed significant treatment effects in the total primary process length: reduction by 32% in CA1 ($p<0.05$) by 50% in CA3 ($p<0.001$) and by 25% in DG ($p<0.01$) (Figure 4.3). Changes were also observed in the primary process mean length: reductions by 25% in CA1 ($p<0.01$), by 45% in CA3, ($p<0.001$) and 25% in DG ($p<0.01$) (Figure 4.4). No treatment effects were seen in the number of primary processes (Figure 4.5).

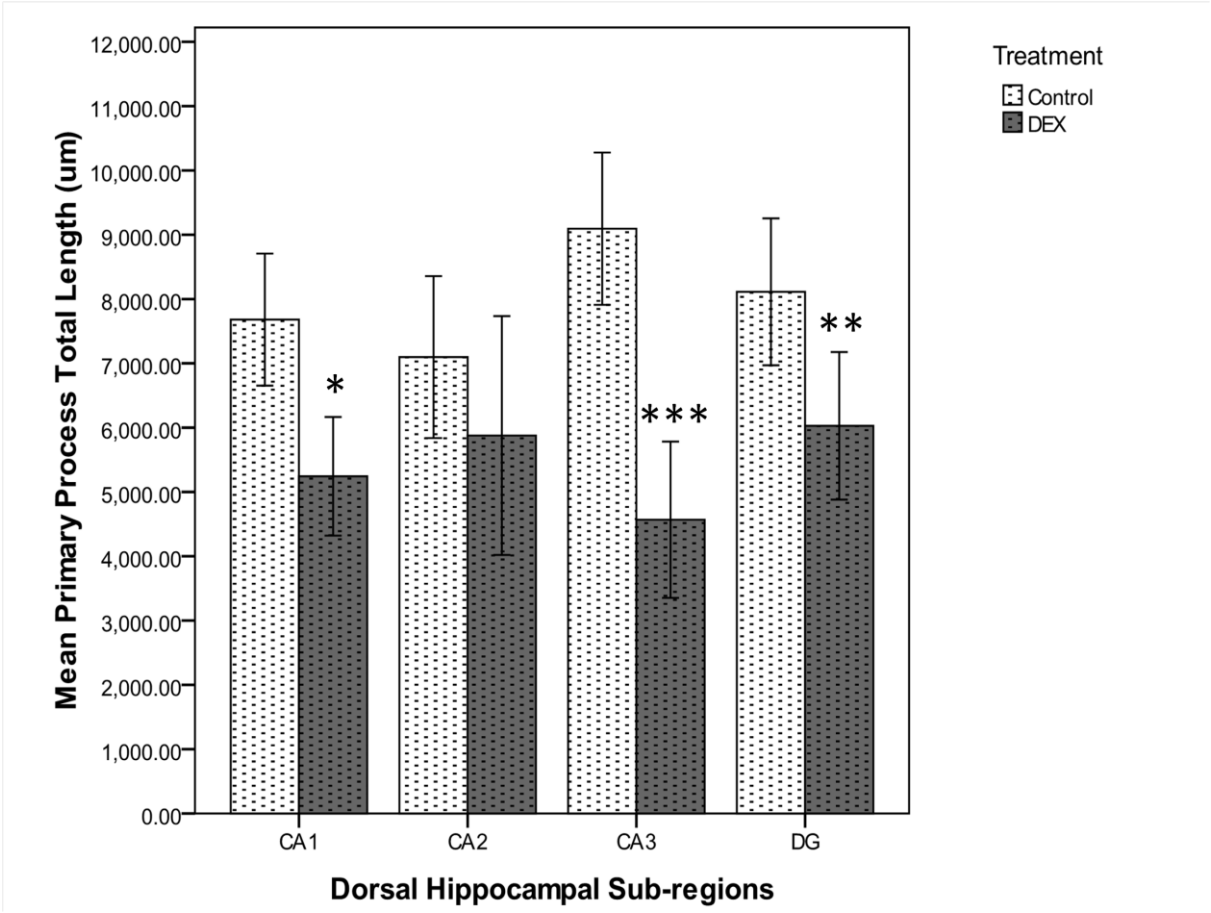

Figure 4.3 – Effect of prenatal dexamethasone treatment on the total primary process length in dorsal hippocampal sub regions in male adult Sprague Dawley rats.

Data are expressed as mean ± SEM (n = 6 per group). *p<0.05, **p<0.01, ***p<0.001

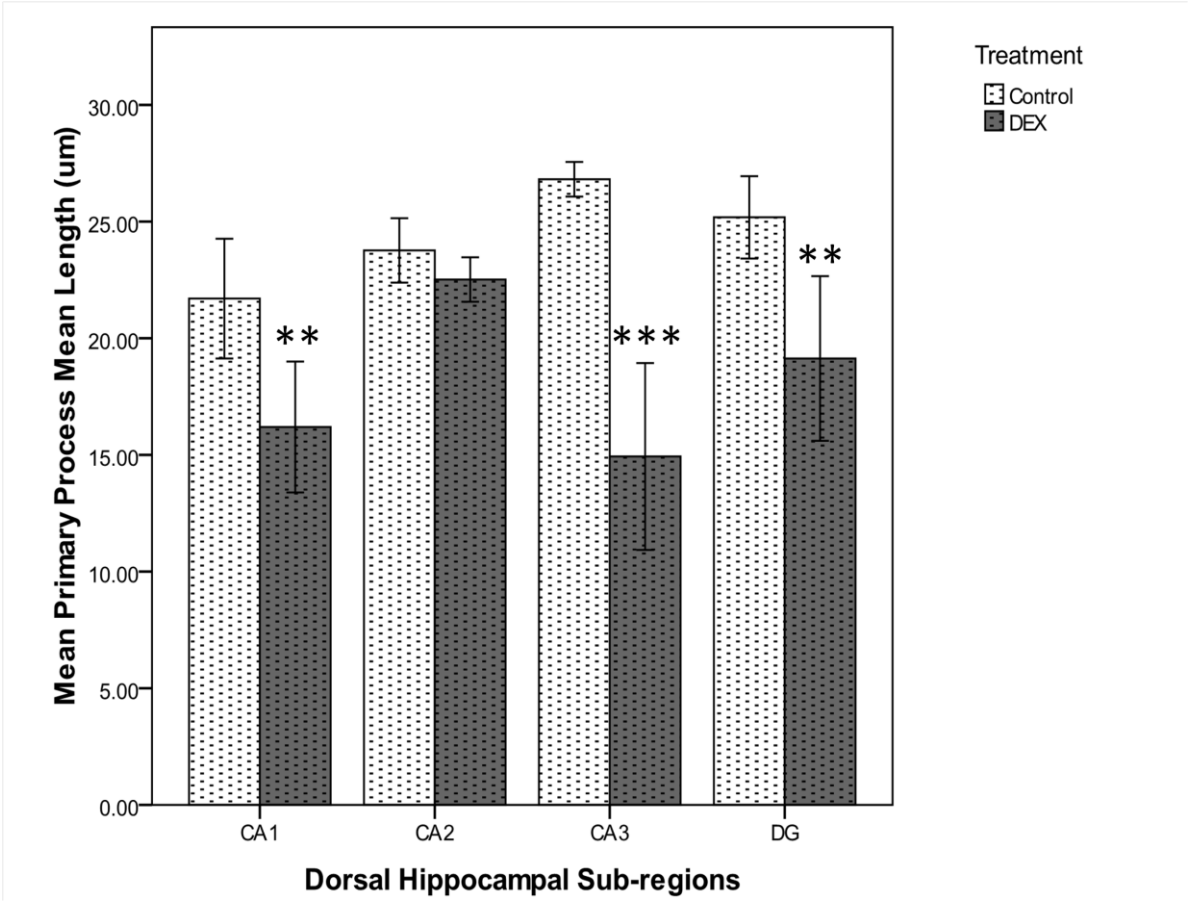

Figure 4.4 – Effect of prenatal dexamethasone treatment on the mean primary process length in dorsal hippocampal sub regions in male adult Sprague Dawley rats. Data are expressed as mean ± SEM (n = 6 per group). **p<0.01, ***p<0.001

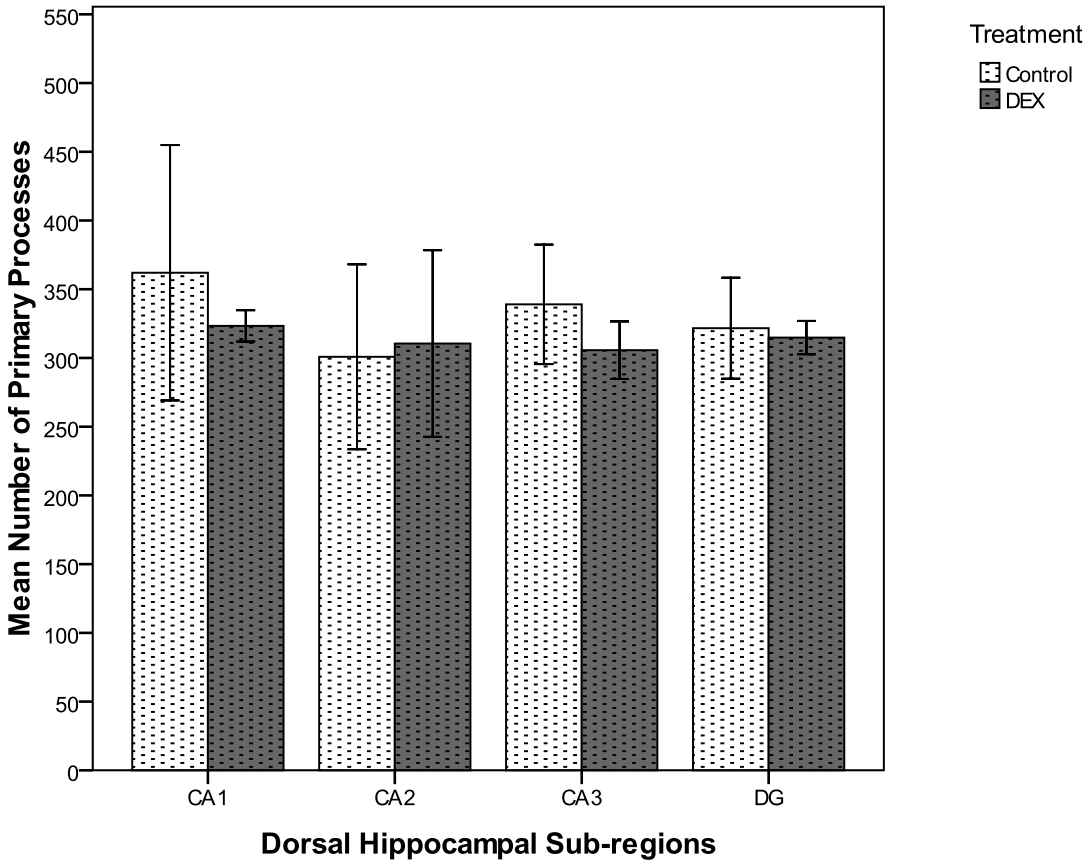

Figure 4.5 – Effect of prenatal dexamethasone treatment on the number of primary processes in dorsal hippocampal sub regions in male adult Sprague Dawley rats.

Data are expressed as mean ± SEM (n = 6 per group).

Morphological analysis of the GFAP positive astroglia revealed no significant changes in the total primary process length (p= 0.674), primary process mean length (p= 0.808) and number of primary processes (p= 0.566) in the basolateral amygdala region (independent t-test analysis, Table 4.5).

GFAP - astroglia morphology		
	Control	Dex Treated
Total primary process length (µm)		
Basolateral amygdala	5761.8 ± 582.6	6123.5 ± 598.9
Nucleus accumbens	4033.4 ± 403.9	3890.1 ± 489.2
Primary process mean length		
Basolateral amygdala	26.8 ± 1.6	27.5 ± 2.1
Nucleus accumbens	22.7 ± 1.8	20.8 ± 1.7
No. of primary process		
Basolateral amygdala	213 ± 11.4	221 ± 9.1
Nucleus accumbens	178 ± 8.5	185 ± 10.6

Table 4.5 – Effects of prenatal dexamethasone exposure on the morphology of GFAP-positive astroglia in male adult Sprague Dawley ratsin the basolateral amygdala and nucleus accumbens region.

Data are expressed as mean ± SEM. (n = 6 per group)

Morphological analysis of the GFAP positive astroglia revealed no significant changes in the total primary process length (p= 0.826), primary process mean length (p= 0.470) and number of primary processes (p= 0.593) in the nucleus accumbens region (independent t-test analysis, Table 4.5).

4.3 Discussion

The present study revealed that prenatal exposure to dexamethasone induced a selective hippocampal volume reduction with no evidence of cell loss accounting for it. With the use of stereology, changes in astroglial morphology were observed, which may underpin the observed hippocampal volume reduction. To my knowledge, this is the first stereology approach to the analysis of anatomical brain changes caused by prenatal dexamethasone in adult rats. Stereology is the method of choice for evaluating changes in regional volume and cell numbers as it offers an unbiased approach to volumetry and morphometry.

As mentioned in the Chapter 3, stress or emotional trauma in early life is associated with risk of depression (Watson et al., 1999; Heim et al., 2004) and schizophrenia (Howes et al., 2004) later in life. Similar risks are also expected to develop in cases of childhood sexual and physical abuse (McCauley et al., 1997) and psychological stressor (Huttunen et al., 1994). Autism (Kinney et al., 2008a) and attention-deficit hyperactivity disorder (ADHD) (Li et al., 2010) are also associated with prenatal stress. Animal studies have compared prenatal dexamethasone treatment with neurobehavioural models of stress and depression-like behaviours (Gregus et al., 2005) with observations such as enhanced fear (Corodimas et al., 1994), reduced body weight (Barr et al., 2000; Kalynchuk et al., 2004), glucocorticoid receptor downregulation (Vyas et al., 2002), HPA dysfunctioning (Pryce et al., 2011) and decreased hippocampal neurogenesis (Pham, 2003). The corticosteroid treated animals possess a major advantage due to less variability in comparison with physical stress models, which avoids individual differences in HPA axis regulation (Gregus et al., 2005).

4.3.1 Effects of dexamethasone treatment on brain weight and volume

Various neurobiological studies are focused mainly on specific brain regions but overall brain size, sometimes measured by weight and sometimes by volume, is also an essential factor which needs to be assessed. The present application of pQCT to measure total brain volume is of relevance to volumetric studies on neuroplasticity where regional volumes need to be scaled to the total brain volume.

Glucocorticoid exposure during pregnancy often leads to a reduction in birth weight in animal models, including primates and humans (Bloom et al., 2001; Newnham et al., 2001). However, another study reported no significant effect on the brain weight and growth of the preterm delivered babies treated with single and multiple courses of antenatal corticosteroids (Murphy et al., 2001). Similar observations were seen in the rats exposed to single betamethasone treatment (Yossuck et al., 2006).

Perinatal corticosteroid exposure is also correlated with a decrease in brain weight. A single dose of betamethasone to pregnant sheep at about 70% of gestation resulted in a reduction in the brain weight by 10% at term and multiple doses resulted into 21% reduction (Huang et al., 1999). Significant brain weight reduction was also reported in rhesus monkeys treated prenatally with corticosteroids (Uno et al., 1990). Repeated doses of dexamethasone on E17–19 resulted in a significant brain weight reduction of the newborn rats (Carlos et al., 1992). In the present study no differences in brain weight were observed between dexamethasone treated and control group.

The brain volume of the adolescents who were born very preterm (i.e. born before 33 weeks) was reduced by 6% (Nosarti et al., 2002). Another MRI study in primates, revealed no significant differences in the brain volume between DEX and vehicle group (Uno et al., 1994). The present study also found no treatment effects on the whole brain volume as measured by pQCT. One of the factors which can affect the brain weight and volume is the amount of corticosteroid administration; in the present study only a minimal dose was given.

4.3.2 Effects of dexamethasone treatment on regional brain volume

Reductions in the regional brain volume in areas such as hippocampus, amygdala and corpus callosum are reported in the premature born children (Abernethy et al., 2002; Isaacs et al., 2000). Smaller amygdala, hippocampus, cerebellum, basal ganglia and corpus callosum were also found in 25 cases of 8 year old preterm children (Peterson et al., 2000). Cortical brain volume decrease by 35% was exhibited in premature infants treated with dexamethasone relative to full term controls (Murphy et al., 2001). On the other hand, Yossuck et al. reported no effect of single betamethasone antenatal dose on regional brain volumes, particularly limbic system (Yossuck et al., 2006).

One of the key regions affected by both perinatal and postnatal exposure to corticosteroids is the hippocampus, which develops primarily during foetal period (Rice and Barone, 2000). Hippocampal atrophy and volume loss are seen following administration of corticosteroids in monkeys and rats (Woolley et al., 1990; Sapolsky et al., 1990). Human adolescents who were born very preterm have been found to

have hippocampal volume loss by 15.6% in the right and 12.1% in the left sides (Nosarti et al., 2002).

The present study reports a significant 8% hippocampal volume loss (expressed as % of total brain volume) in dexamethasone treated rats when compared with controls, even though the total brain volume remains unaffected. No changes were seen in the basolateral amygdala and nucleus accumbens volume, which indicates a selective treatment effect on the hippocampus. Hippocampus is the region where neuronal cell proliferation is pronounced and its volume loss might manifest in impaired hippocampus-dependent memory functioning.

The present reduction of the hippocampal volume is moderate but statistically significant. Previous publications report a wide range of losses in the hippocampal volume across several species. Thus studies have demonstrated an overall 30% reduction in the hippocampal volume in the 9 month old juvenile monkeys treated with DEX which was later confirmed with MRI study, for long term effect, indicating similar volume changes after 2 years (Uno et al., 1994). Coe et al. also found hippocampal volume decrease by 12% and 10% in the offspring of rhesus monkeys exposed to early (GD 50 to 92) and late (GD 105 to 147) natural acoustic startle stressor, respectively (Coe et al., 2003). In animals, four weeks of cortisol treatment or psychosocial stress resulted in hippocampal volume loss by 5-10% (Ohl et al., 2000). Chronic social defeat stress also showed mild but significant hippocampal volume shrinkage in adult rats (Czeh et al., 2010).

4.3.3 Effects of dexamethasone treatment on cell numbers and morphology

The present findings indicate no significant treatment effects on the total cell counts, which suggests no cell loss as such, although it is theoretically possible that unidentified neuronal and/or glial cell losses might account for the hippocampal shrinkage.

The present results are consistent with the published animal studies on effects of cortisol or chronic stress exposure, using a stereological approach, which showed no hippocampal cell loss (Leverenz et al., 1999; Sousa et al., 2002; Vander Beek et al., 2004 and Vollmann-Homsdorf et al., 1997). A human study also reported no evidence of significant cell loss in hippocampus in response to corticosteroid treated and depressed patients (Lucassen et al., 2001a; Muller et al., 2001).

The present study focused on the effect of dexamethasone treatment on the astroglia cell numbers and morphology. To my knowledge this is the first study to report the data on astroglial cell count and morphology estimated by means of stereology in adult rats that were prenatally treated with dexamethasone. Although the most consistent finding in animals treated with glucocorticoid or exposed to chronic stress is a selective reduction in volume of the hippocampus (Fuchs and Flugge, 2003), in the studies on the mechanisms underlying the glucocorticoid-induced volume reductions, the attention has been focused on neurones. As a result, neuronal cell loss is well researched and documented and various dexamethasone treatments were found to lead to neuronal loss in rats (Haynes et al., 2001; Sousa et al., 1999) and primates (Uno et al., 1990; Sapolsky et al., 1990) mostly in the hippocampal CA3

and dentate gyrus. However less attention has been given to astroglial cells, the most important non-neural cells implicated in the maintenance of brain homeostasis.

Inhibition of glial cell proliferation in response to exogenous glucocorticoids or stress were reported in *in vitro* (Crossin et al., 1997; Sabolek et al., 2006) and *in vivo* (Wennstrom et al., 2006) studies. GFAP is an intermediate filament protein that provides support for cell structure and movement, cell communication, and blood–brain barrier (BBB). Other studies also suggest that treatment with corticosterone or synthetic glucocorticoids such as dexamethasone inhibit GFAP expression in the neonatal and adult rat brain (Nichols et al., 1990a,b; Laping et al., 1991; Tsuneishi et al., 1991 and O'Callaghan et al., 1991) suggesting that astrocytes can be affected by glucocorticoid overload. In a clinical context, post mortem studies in depressed patients have reported reductions in the numbers of glial cell in the prefrontal cortex and amygdala brain regions (Rajkowskal., 2000; Bowley et al., 2002).

Hippocampal volume losses could be also caused by impoverished morphology of neurones and/or glial cells as they respond to environmental stimuli. There are several animal studies that have documented neuronal dendritic shrinkage after corticosterone administration or chronic stress (Magarinos et al., Sousa et al., 2000; 1996; Wolley et al., 1990; Watanabe et al., 1992 and Morales Medina et al., 2009). However, one study observed short term effect in the dendritic reduction (granule cells 35%, CA3 25% and CA1 13-20%) which showed improvement in the structure later on after the recovery which correlated with marked improvement in behaviour (Sousa et al., 2000). In support of astroglial plasticity, one of the recent studies

reported an increase in astrocytic primary process length in response to the enriched environment (Violaa et al., 2009).

It is now evident that non-genetic/environmental factors acting in early life result in permanent alterations of the physiological systems. Perinatal programming and/or plasticity of the physiological system in extreme conditions like stress or foetal corticosteroid exposure may result in long term abnormalities (Barker et al., 1993; Maccaria et al., 2003). Interestingly, similar long-term effects on the adult brain were observed in postnatally deprived rats (Shende et al., 2011) suggesting that exposure to stressors either prenatally or postnatally regulates the long term brain functioning, and in humans is a major aetiological factor in neurological disorders (Pryce et al., 2011).

4.4 Conclusions

To conclude, the present stereological analysis of GFAP-positive astroglia showed significant changes in the total and mean primary process lengths in the hippocampal subregions CA1, CA3 and dentate gyrus, as a long-term effect of prenatal exposure to dexamethasone. It is plausible to assume that these changes contribute to the losses in the hippocampal volume found in the same animals. The observed dexamethasone-induced impoverishment of astroglial morphology can be treated as a demonstration of glial plasticity, a phenomenon that deserves more research in the context of effects of corticosteroid overload and stress-related pathologies.

These lines of evidence support the concept that the perinatal period is indeed a sensitive window for neuroplastic changes, which may lead to heightened cell vulnerability by determining permanent changes in the expression of molecular regulation of neuroplasticity (primarily neurotrophic factors) and in the neurogenic process. The regional selectivity of these modifications suggests that such vulnerability is not widespread but, instead, affects specific cell populations or neuronal pathways. This is of potential relevance to stress-related psychiatric disorders.

Chapter 5

Prenatal exposure to glucocorticoids: effects of dexamethasone treatment on brain neuromodulatory systems – oxytocin and serotonin

5.1 Introduction

As discussed in the Chapters 1 and 4, early life experiences especially during the prenatal period, when the nervous system is developing can lead to long term consequences. Prenatal stressors such as psychological stress, malnutrition in later gestation, maternal viral infection were recently linked to schizophrenia (Sullivan, 2005; Lee et al., 2007) and autistic disorder (Beversdorf et al., 2005). The various types of experimental models of prenatal stress include, but are not limited to, *in utero* glucocorticoid treatment, maternal infection, maternal nutritional deficiency or maternal exposure to psychological stress (Meyer and Feldon, 2010). The most common animal model widely studied/used for prenatal stressful manipulation is the foetal exposure to corticosteroids (Hauser et al., 2006, 2008, 2009; Slotkin et al., 2006).

Prenatal exposure to high levels of glucocorticoids affects neuronal cell sensitivity and apoptosis of cerebellar granule cells to specific stimuli, such as oxidative stress (Ahlbom et al., 2000). Experimental rats exposed to prenatal stress (exposure to bright light) showed decrease in the hippocampal granule neuronal cell proliferation, decreased BDNF levels (Van Den Hove et al., 2006) and deficits in the learning tasks (Lemaire et al., 2000). Altered BDNF and TrkB mRNA expression was also observed in response prenatal dexamethasone exposure in rats (Hossain et al., 2008).

Research published on prenatal stress has reported various alterations in behaviour, neuroanatomical architecture, neurochemical functioning and neuroendocrine activity. However, less attention has been paid to the role of neuropeptide systems in prenatal stress. At the same time, neuropeptides, such as oxytocin and vasopressin have been implicated in the regulation of social behaviour (Ferguson et al., 2001;

Donaldson and Young, 2008) and in response to stress (Gimpl and Fahrenholz, 2001). It has been suggested that the timing of the early life stressor either prenatal and/or postnatal is critical to the neurochemical development as it can lead to behavioural abnormalities in later stages of life.

There is consensus that GCs such as cortisol in primates and corticosterone in rodents play an important role in prenatal programming of the brain and neuroendocrine systems. Excessive prenatal exposure to GCs, either endogenous under conditions of sustained stress, or exogenous during e.g. dexamethasone treatment, can lead to long-term dysregulation of GC receptors and impairment of the feedback mechanism, which controls the HPA axis and its reactivity to stress. Such dysregulation can have long-term neuro-behavioural implications (Welberg and Seckl, 2001).

Foetal exposure to GCs results in the reprogramming of the HPA axis and long term changes in the neurotransmitter function (Seckl, 2004). Dexamethasone (Dex) administration during late gestation induces anxiety-like behaviour consistent with excessive sensitivity of HPA axis in male offspring (Nagano et al., 2008) and impairments in startle response (Kleinhaus et al., 2010). The type of above mentioned behavioural changes triggered by prenatal exposure to Dex in children and experimental animals raises a question whether those implicate oxytocin, which plays an important role in the regulation of social behaviour (Ferguson et al., 2001, Gimpl and Fahrenholz, 2001, Donaldson and Young, 2008), anxiety (Windle et al., 2006) and responses to stress (Gimpl and Fahrenholz, 2001). A link between prenatal exposure to DEX and oxytocin is plausible as it has been known that central oxytocin receptors undergo changes in the binding during process of neurodevelopment

(Tribollet et al., 1989).

5.1.1 Effects of prenatal stress on oxytocinergic system

The neuropeptide oxytocin (OT) is synthesised in the paraventricular and supraoptic nucleus of the hypothalamus and released into the blood from the neurohypophysis and centrally within the brain; it is known for its important role in sexual functions, parturition, lactation and maternal behaviour (Gimpl and Fahrenholz, 2001).

Oxytocin receptors are regulated by glucocorticoids in the CNS (Insel and Winslow, 1991; Kremarik et al., 1993). OT is a hypophysiotrophic hormone which under stressful situation controls ACTH release. This oxytocin-induced ACTH secretion is blocked by adrenal glucocorticoid hormone (Gibbs, 1986). Hence, oxytocin regulates the HPA axis functioning by reducing ACTH release which in contrast is helpful in treating stress-HPA induced pathologies (Boutet et al, 2006). Preclinical and clinical studies have shown that the plasma OT concentrations increase under various stressful stimuli such as conditioned fear stimuli, electric foot shocks and restraint stress (Onaka, 2004; Pierrehumbert et al., 2010).

Administration of OT intracerebroventrically is found to regulate sexual behaviour (Argiolas and Gessa, 1991) and decrease in anxiety in male rats (Ring et al., 2006) and anxiolytic effects (Windle et al., 1997) were also observed in female rats. In humans, intranasal administration of OT result into enhanced trust (Kosfeld et al., 2005), social support (Heinrichs et al., 2003), positive social memories (Guastella et al., 2008) and reduction of aggression and anxiety (Huber et al., 2005; Heinrichs and Domes, 2008).

Central oxytocin receptors in the brain are expressed by both neurones and glial cells (Discalaguenot and Strosser, 1995). In the rat brain oxytocin receptors are found in regions such as the limbic system, cortical areas, the basal ganglia and the thalamus (Gimpl and Fahrenholz, 2001).

Studies have reported oxytocin receptor activation in the medial amygdala (Ferguson et al., 2001, Timmer et al., 2011) and intracerebral administration of oxytocin in amygdala of oxytocin-knockout mice (Winslow and Insel, 2002, Lukas et al., 2011) resulting into improved social recognition skills hence suggesting the role of oxytocin in the social cognition. Another study concluded decreased oxytocin levels in the amygdala and paraventricular hypothalamic nucleus mediated via oxytocin receptor alpha and beta involved mechanisms (Choleris et al., 2003).

Association between oxytocin receptor mRNA and its interaction in the regulation of social behaviour in male mice have been confirmed through a gene expression study (Murakami et al., 2011). Oxytocin is found to decrease amygdale-mediated fear response both in humans (Kirsch et al., 2005) and rodents (Viviani et al., 2011) which supports its potential therapeutic use for clinical related fear conditions (Bartz et al., 2011). Oxytocin also facilitates hippocampal neurogenesis and neural proliferation in adult rats following dexamethasone overexposure suggesting its neuroprotective role (Leuner et al., 2011).

Oxytocinergic system can play a role in the pathophysiology of various mental disorders such as autism (Andari et al., 2010), schizophrenia (Rubin et al., 2011) and borderline personality disorders (Simeon et al., 2011). A significant reduction in cortisol levels after the social stress exposure in individuals with low emotional regulation has been observed (Quirin et al., 2011).

A strong interaction between the oxytocinergic system (part of the neurohypophysial system) and HPA axis (regulator of stress system) by means of the bidirectional regulatory mechanism has been suggested (Engelmann et al., 2004). Stressful experiences in the form of foot shock and forced swimming has been found to increase the central oxytocin secretion in both male and female rats (Bruhn et al., 1986). Disruption in the oxytocin activity and learning and memory tasks was observed under the glucocorticoid administration (0.02 mg/kg - intraperitoneal) in rats (Furstenau de Oliveira et al., 2007). Prenatal corticosteroid exposure leads to decrease in the oxytocin mRNA levels in the hypothalamic paraventricular nucleus and an increase in the oxytocin receptor binding in central amygdala (Lee et al., 2007). Oxytocin receptor binding is also reduced in the rats underwent maternal separation (Lukas et al., 2010).

Human studies have also reported altered oxytocin status in relation to early life stress and depressive traits (Heim et al., 2009; Opacka-Juffry and Mohiyeddini, 2011).

Considering the fact that the oxytocinergic system undergoes alterations during the prenatal – foetal nervous system development it is plausible to hypothesise that oxytocin receptor binding will be affected by prenatal dexamethasone exposure (Tribollet et al., 1989). Previous studies also suggest altered oxytocin receptor system was brain region dependent in response to early life stress experiences (Lee et al., 2007).

5.1.2 Effects of prenatal stress on serotonergic system

There is strong correlation between the brain serotonin system and the HPA axis and alteration in any one of them can result in functional impairment (e.g. Holmes et al., 1995). These systems are found to be impaired by stress and/or depression (Bhagwagar et al., 2002).

As mentioned earlier, links between prenatal corticosteroid exposure and various psychological, behavioural and cognitive disorders are demonstrated in animal experiments (Huizink et al., 2002, Charil et al., 2010). Studies have reported changes in the glucocorticoid receptor expression which in turn leads to HPA axis destabilisation (Takahashi et al., 1991; Shoener et al., 2006) in rats exposed to high synthetic glucocorticoid levels. The HPA axis activity is also regulated by the serotonergic system (Klaassen et al., 2002).

Serotonin or 5- hydroxyl tryptamine (5-HT) is the key regulator in various forms of behaviours including sleep, mood and emotions (Stockmeier, 2003; Slotkin et al., 1996). Serotonin pathways and receptors are involved in the aetiology of depression and its treatment, with the 5HT-1A receptor being most likely implicated (Hoyer et al., 2002; Stockmeier, 2003).

5HT-1A receptors exist as presynaptic somatodendritic autoreceptor mainly found in the raphe nuclei and postsynaptic heteroreceptor mainly found in cortex and limbic system (Drevets et al., 2007). The presynaptic autoreceptors mainly function to inhibit neuronal firing and reduce serotonin release (Celada et al., 2001). Both the pre and postsynaptic 5HT-1A receptors have a substantial effect on the stress and anxiety as summarised below.

Exposure to stress and glucocorticoids has prominent effects on the expression of the 5HT-1A receptor expression (Leonard, 2005). Over-expression and increased production of 5HT-1A receptor mRNA is seen around the gestational day (GD) 12 in rats (Hillion et al., 1993) and high concentration of 5HT-1A receptor binding in the human embryonic brain is observed in the cortex and hippocampus at the age of 16 - 22 week during pregnancy (Olmo et al, 1998, Andrews et al., 2004). During depression both the HPA axis and the serotonergic system is found to be abnormal (Slotkin et al., 1996).

It has been suggested that an increase in the presynaptic 5HT-1A receptor in the raphe nuclei can act as an anti-anxiety factor (Andrews et al., 1994) but increase in the postsynaptic 5HT-1A receptor located in the hippocampus and amygdala may lead to anxiety (Holmes et al., 1994). The deletion of the 5HT-1A receptor in mice showed an increase in the anxiety like behaviour in the open field and novelty suppressed feeding paradigms suggesting the importance of the 5HT-1A receptor (Gross et al., 2000). Presynaptic 5HT-1A receptors inhibit the tyrosine hydroxylase synthesis which can result in serotonergic suppression (Stockmeier, 2003). Experimental animal studies have also reported an increase in the serotonin turnover in the nucleus accumbens, hypothalamus and amygdala regions under stress exposure (Holmes et al., 1994). Serotonin receptor sensitivity and density appear to be affected by anxiety (Harvey et al., 2003).

The 5HT-1A receptor is the main element of the serotonin system which is involved in the aetiology and treatment of depression (Hoyer et al., 2002; Savitz et al., 2009). 5HT-1A receptors are found majorly in the limbic system of the brain in particular in the hippocampus (Andrews et al., 2003). Reduction in the 5HT-1A receptor was seen

in the patients suffering from depression (e.g. Drevets et al., 2007) along with high levels of glucocorticoid hormones (Holmes et al., 1995; Stockmeier, 2003). Glucocorticoid exposure results in altered 5HT-1A receptor binding which could be the possible reason to the less therapeutic response of the serotonin receptor agonist in the depression (Savitz et al., 2009).

5.1.3 Aims and Hypotheses

The present study aimed to investigate the long term effects of prenatal dexamethasone administration on the oxytocin and 5HT-1A serotonin receptor binding in male and female rats. Brain regions i.e. amygdala, hippocampus, nucleus accumbens, ventromedial hypothalamus, lateral septum and raphe nuclei were investigated considering their involvement in the key processes like memory and learning, stress related conditions, fear, social recognition, reward and pleasure and sexual behaviour; where oxytocinergic system also plays a regulatory role in these processes (Insel and Young, 2000; Gimpl and Fahrenholz, 2001; Kirsch et al., 2005; Ross and Young, 2009). Also, the fact that very few studies have researched the prenatal period, as most of the reported studies have analysed the postnatal period, motivates the present study.

The specific hypotheses tested were as follows:

- Prenatal dexamethasone exposure will result in a significant reduction in the oxytocin receptor binding in the limbic brain regions.
- Prenatal dexamethasone exposure will decrease the 5HT-1A receptor binding in the brain regions that regulate stress and depression like behaviour in rats.

5.2 Results

5.2.1 Oxytocin receptor binding

The autoradiograms of the brain sections labelled with [^{125}I] OVTA demonstrate a high density of oxytocin receptors in the amygdala, subiculum, nucleus accumbens, septal nuclei, ventromedial hypothalamic nuclei and hippocampal subregions (e.g. shown in Figure 5.1).

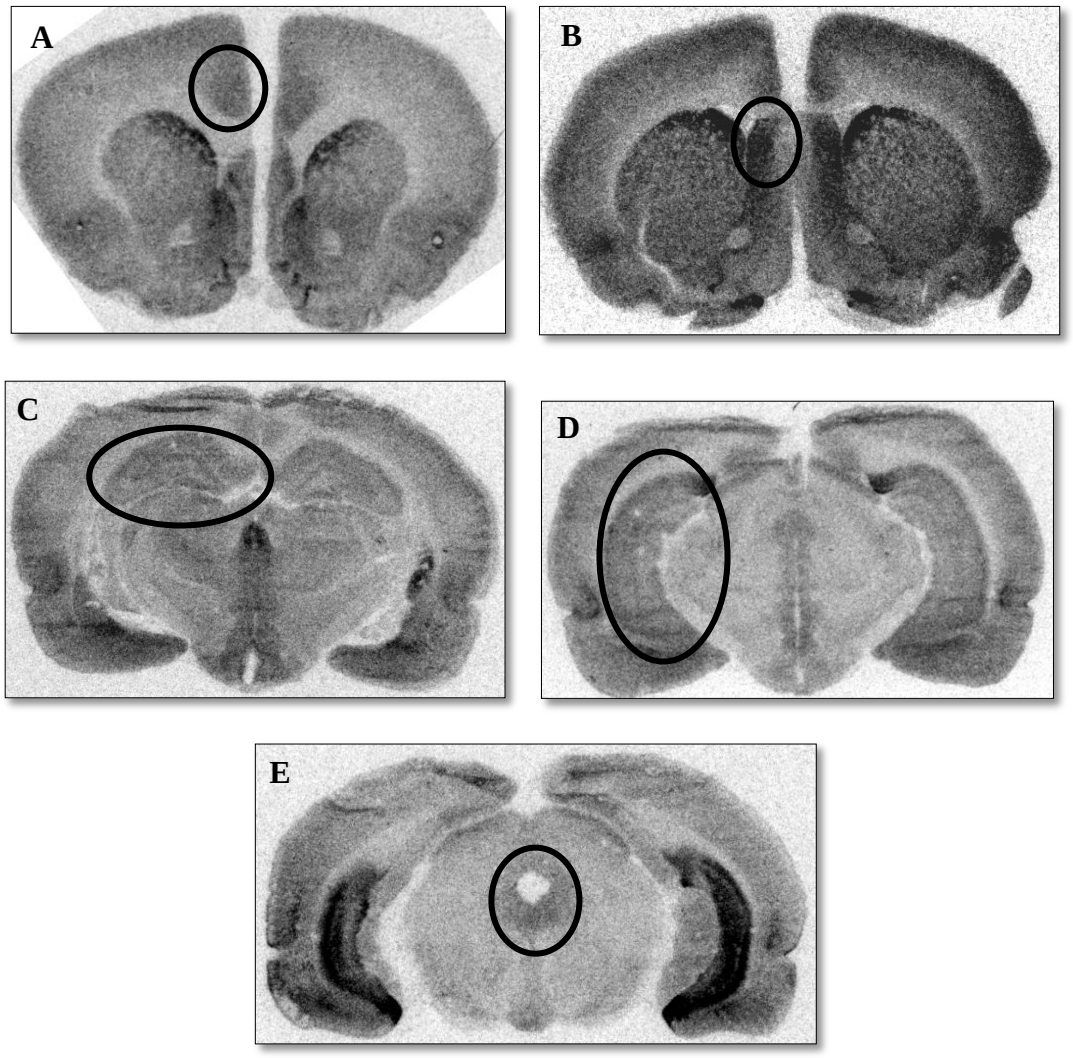

Figure 5.1 – Autoradiograms of Dex treated coronal female rat brain sections labelled with [^{125}I] OVTA ligand representing the frontal cortex (A), lateral septal nuclei (B), dorsal hippocampus (C), ventral hippocampus (D) and raphe nuclei (E) regions.

Receptor binding values were higher in control females when compared with control males in the prelimbic cortex ($F=38.38$, $p<0.001$), lateral septum ($F=11.834$, $p<0.01$), nucleus accumbens ($F=22.60$, $p<0.001$), basolateral amygdala ($F=39.93$, $p<0.001$), central amygdaloid nucleus ($F=66.68$, $p<0.001$), ventromedial hypothalamus ($F=35.61$, $p<0.001$), dorsal hippocampal CA2 ($F=5.15$, $p<0.05$), dentate gyrus ($F=12.87$, $p<0.01$) and raphé nucleus ($F=10.27$, $p<0.01$). Interestingly, control males also showed significantly higher oxytocin receptor binding compared to females in the subiculum transition area ($F=10.\ 8$, $P<0.01$) and ventral hippocampal CA1 ($F=29.\ 2$, $P<0.001$), CA2 ($F=79.\ 45$, $P<0.001$) and CA3 ($F=10.\ 52$, $P<0.01$).

Significant reduction in the oxytocin receptor binding was observed in ventral hippocampus (CA1, CA2, and CA3) and PVN region in males and LSD and VMH in females. However, a significant increase was seen in the dorsal hippocampal CA2 and PVN regions in females only.

A significant main effect of treatment was observed in the basolateral amygdala ($F=9.\ 34$, $p<0.01$), and a significant main effect of sex in BLA ($F=57.\ 86$, $p<0.001$) and central amygdala (CEM) ($F=72.\ 1$, $p<0.001$) was found. The sex x treatment interaction analysis revealed no significant changes in BLA ($F=0.003$, $p>0.05$) and CEM ($F=0.71$, $p>0.05$) regions (Table 5.1). In terms of the OTR binding, no significant changes were seen in BLA (males - 16%↑, $p>0.05$ & female - 11%↑, $p>0.05$) and CEM (males - 3%↓, $p>0.05$ & female - 11%↓, $p>0.05$) regions (Figure 5.2).

In the present study, post hoc analysis indicated significant reductions in the oxytocin receptor (OTR) binding in LSD in Dex treated females (27%, $p<0.05$) and no effect was seen in males (11%, $p>0.05$) when compared with control. There was also a

significant main effect of sex (F=15.31, p<0.01) and treatment (F=7.01, p<0.05) in the LSD region.

Univariate ANOVA analysis showed no significant main effect of treatment in the prelimbic cortex (PrL) (F=0.69, p>0.05) and nucleus accumbens (NAcc) (F=0.02, p>0.05) regions. However, a significant main effect of sex was seen in the PrL (F=94.11, p<0.001) and NAcc (F=40.69, p<0.001) regions. There was also a significant reduction in OTR binding in the NAcc region in DEX males (23%, p<0.01) but not females, compared to controls.

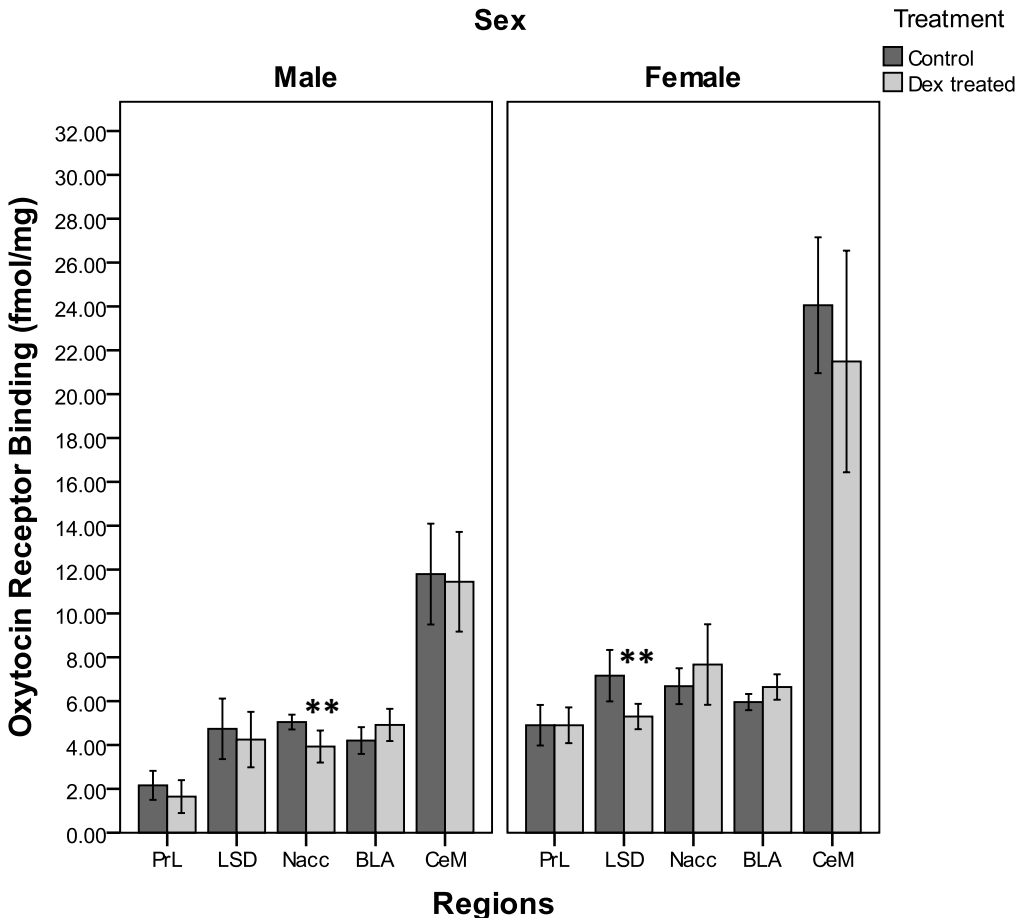

Figure 5.2 - Oxytocin receptor binding in the prelimbic cortex (PrL), lateral dorsal septum (LSD), nucleus accumbens (NAcc), basolateral amygdala (BLA) and central

amygdala (CEM) in male and female rats in response to prenatal dexamethasone exposure.

Data expressed as mean values ± SEM in fmol/mg; n = 6 per group.

**p<0.01 DEX versus control

The observed results in the dorsal hippocampal sub areas show significant main effects of sex: CA1 ($F=8.75$, $p<0.01$), CA2 ($F=9.81$, $p<0.01$) and DG ($F=28.45$, $p<0.001$). In DEX-treated females, there was a significant increase in the OTR binding in the CA2 region (67%, $p<0.001$ versus control). No changes in the OTR binding were observed in the other dorsal hippocampal subregions of DEX treated males when compared with controls (Figure 5.3). There were significant sex x treatment interactions in CA2 ($F=28.35$, $p<0.001$) and CA3 ($F=5.04$, $p<0.05$) with no effects in CA1 ($F=4.11$, $p>0.05$) and DG ($F=0.49$, $p>0.05$) (Table 5.1).

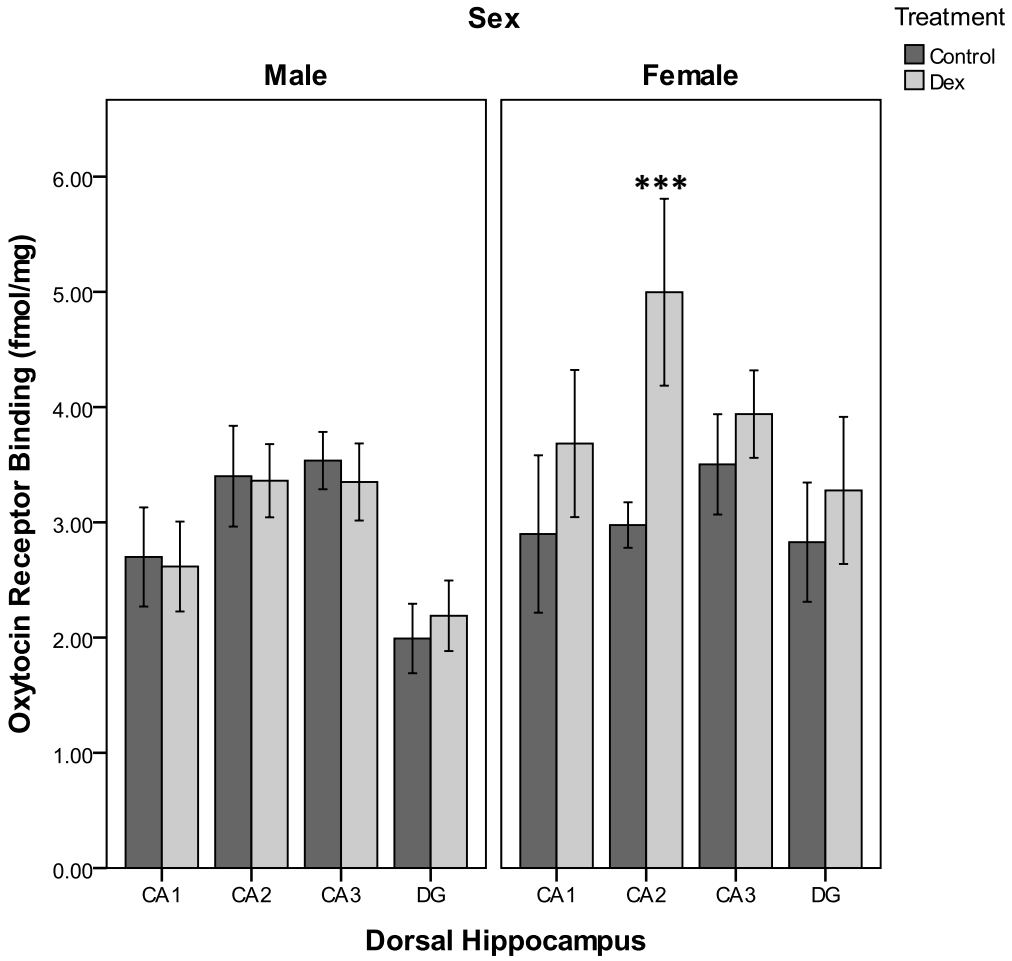

Figure 5.3 - Effect of prenatal dexamethasone exposure on the oxytocin receptor binding in the dorsal hippocampal sub regions in male and female rats.

Data expressed as mean values ± SEM in fmol/mg; n = 6 per group.

***p<0.001 DEX versus control

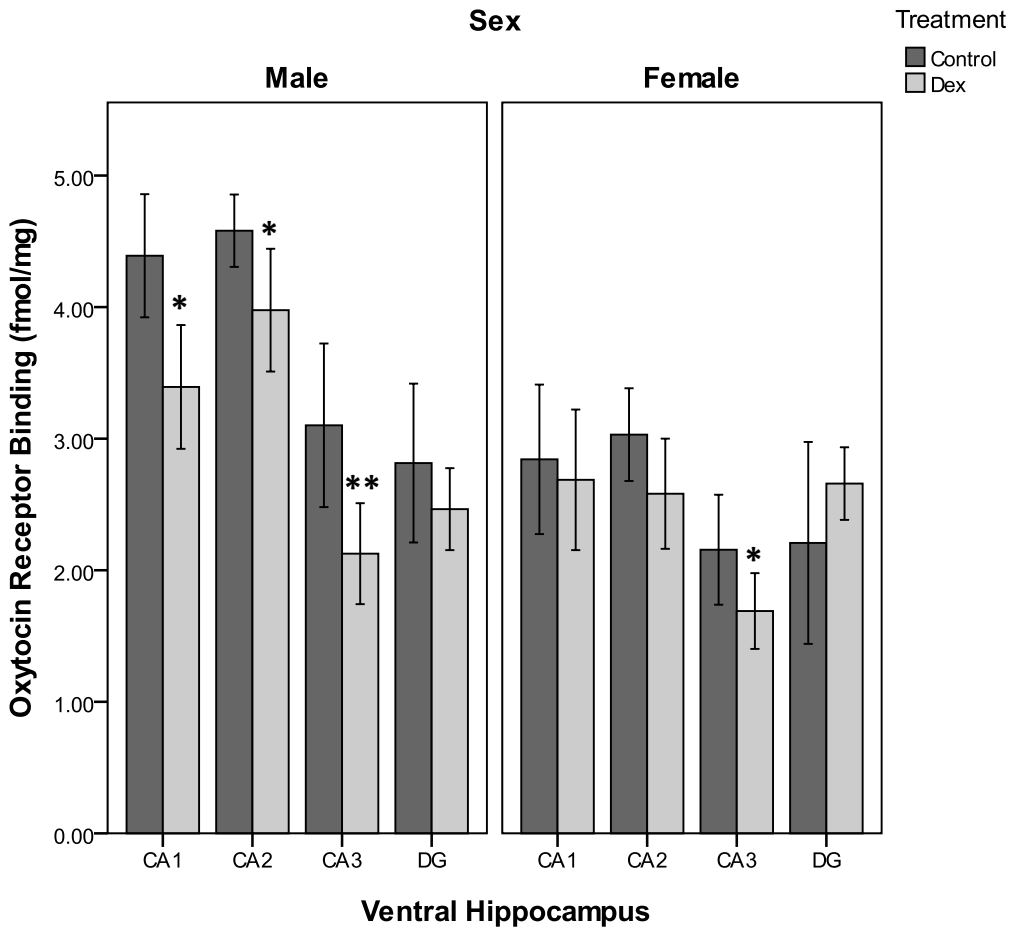

Figure 5.4 - Effect of prenatal dexamethasone exposure on the oxytocin receptor binding in the ventral hippocampal sub regions in male and female rats.

Data expressed as mean values ± SEM in fmol/mg; n = 6 per group.

*p<0.05 **p<0.01 DEX versus control

Significant main effects of sex were observed in the ventral hippocampal CA1 ($F=31.99$, $p<0.001$), CA2 ($F=96.68$, $p<0.001$) and CA3 ($F=15.94$, $p<0.01$). The OTR binding in DEX-treated males showed significant reductions in CA1 (40%, $p<0.05$), CA2 (25%, $p<0.05$) and CA3 (38%, $p<0.01$) with no changes in DG, when compared with controls. In DEX-treated females, a significant reduction in the OTR binding was observed in CA3 (22%, $p<0.05$) with no significant treatment effects in CA1, CA2 and DG when compared with the control (Figure 5.4). There was a significant

interaction between Sex x Treatment in CA1 ($F=4.46$, $p<0.05$) with no effects in CA2 ($F=0.27$, $p>0.05$), CA3 ($F=2.17$, $p>0.05$) and DG ($F=3.77$, $p>0.05$) (Table 5.1).

A significant main treatment effect was observed in the ventro-medial hypothalamus ($F=7.08$, $p<0.05$). A significant reduction was seen in the OTR binding in DEX-treated females vs control (25%, $p<0.01$) in the VMH region with no treatment effect in males (0.5%, $p>0.05$) in the VMH region ($F=6.84$, $p<0.05$) (Figure 5.5).

In addition, there was a significant sex x treatment interaction in the PVN ($F=27.42$, $p<0.001$) with no effects observed in STR ($F=0.33$, $p>0.05$) and raphé nuclei ($F=0.003$, $p>0.05$, Table 5.1).

No significant main effect of treatment was observed in the paraventricular nucleus (PVN) ($F=1.13$, $p>0.05$), subiculum transition area ($F=0.18$, $p>0.05$) and raphé (both dorsal and ventral) ($F=0.09$, $p>0.05$). A significant main effect of sex was found in PVN ($F=24.49$, $p<0.001$), subiculum transition area ($F=19.69$, $p<0.001$) and raphé ($F=17.73$, $p<0.001$) (Table 5.1). There were significant differences between DEX and control groups: OTR binding was significantly reduced in the PVN in males (by 45%, $p<0.01$) but increased in females (by 29%, $p<0.05$) (Figure 5.5).

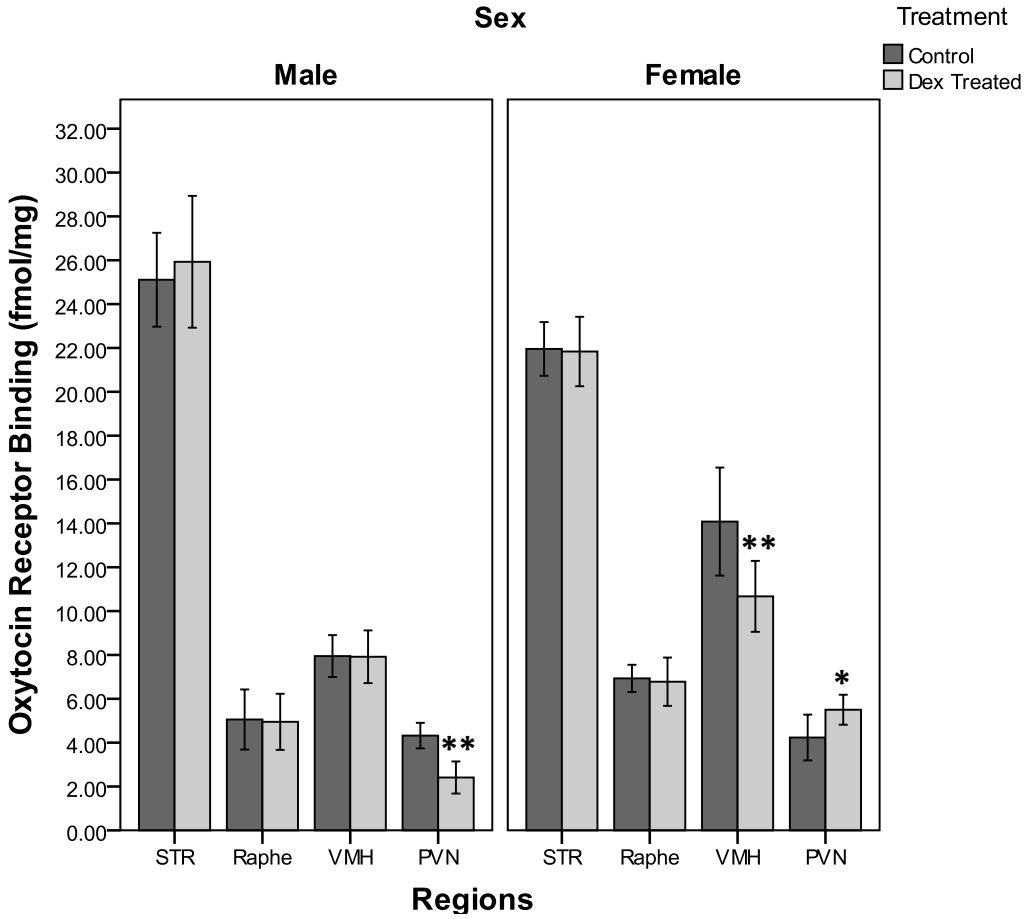

Figure 5.5 - Oxytocin receptor binding in the subiculum transition area (STR), raphe nuclei, ventromedial hypothalamic nucleus (VMH) and paraventricular hypothalamic nucleus (PVN) in male and female rats in response to prenatal dexamethasone exposure.

Data expressed as mean values ± SEM in fmol/mg; n = 6 per group.

*p<0.05, **p<0.01 DEX versus control

Table 5.1 - presents the results of a univariate analysis of variance between sex and treatment in oxytocin receptor binding.

	Brain Region		Source of variation	$F_{value - F(1,20)}$	P_{value}
Oxytocin Receptor binding	LSD		Sex	15.305	P < 0.01 **
			Treatment	7.015	P < 0.05 *
			Sex x Treatment	2.389	P > 0.05
	PrL		Sex	94.108	P < 0.001 ***
			Treatment	0.694,	P > 0.05
			Sex x Treatment	0.682,	P > 0.05
	Nacc		Sex	40.690	P < 0.001 ***
			Treatment	0.024	P > 0.05
			Sex x Treatment	6.229	P < 0.05 *
	BLA		Sex	57.864	P < 0.001 ***
			Treatment	9.338	P < 0.01 *
			Sex x Treatment	0.003	P > 0.05
	CEM		Sex	72.096	P < 0.001 ***
			Treatment	1.231	P > 0.05
			Sex x Treatment	0.710	P > 0.05
	VMH		Sex	47.280	P < 0.001 ***
			Treatment	7.084	P < 0.05 *
			Sex x Treatment	6.835	P < 0.05 *
	PVN		Sex	24.494	P < 0.001 ***
			Treatment	1.126	P > 0.05
			Sex x Treatment	27.416	P < 0.001 ***
	STR		Sex	19.686	P < 0.001 ***
			Treatment	0.184	P > 0.05
			Sex x Treatment	0.329	P > 0.05
	Raphe		Sex	17.732	P < 0.001 ***
			Treatment	0.085	P > 0.05
			Sex x Treatment	0.003	P > 0.05
	Dorsal Hippocampus	CA1	Sex	8.749	P < 0.01 **
			Treatment	2.690	P > 0.05
			Sex x Treatment	4.109	P > 0.05
		CA2	Sex	9.810	P < 0.01 **
			Treatment	26.231	P < 0.001 ***
			Sex x Treatment	28.347	P < 0.001 ***
		CA3	Sex	4.030	P > 0.05
			Treatment	0.816	P > 0.05
			Sex x Treatment	5.039	P < 0.05 *
		DG	Sex	28.446	P < 0.001 ***
			Treatment	3.211	P > 0.05
			Sex x Treatment	0.488	P > 0.05
	Ventral Hippocampus	CA1	Sex	31.996	P < 0.001 ***
			Treatment	8.376	P < 0.01 **
			Sex x Treatment	4.461	P < 0.05 *
		CA2	Sex	96.681	P < 0.001 ***
			Treatment	12.364	P < 0.01 **
			Sex x Treatment	0.266	P > 0.05
		CA3	Sex	15.941	P < 0.01 **
			Treatment	17.347	P < 0.001 ***
			Sex x Treatment	2.168	P > 0.05
		DG	Sex	0.999	P > 0.05
			Treatment	0.060	P > 0.05
			Sex x Treatment	3.765	P > 0.05

5.2.2 5HT-1A receptor binding

Tritium-labelled WAY100635 is a selective 5HT-1A antagonist radioligand used for *in vitro* (Khawaja et al., 1995) and *in vivo* (Hume et al., 1994) studies. The present study employed the same ligand to identify 5HT-1A receptors by autoradiography technique (as Leventopoulos et al., 2009).

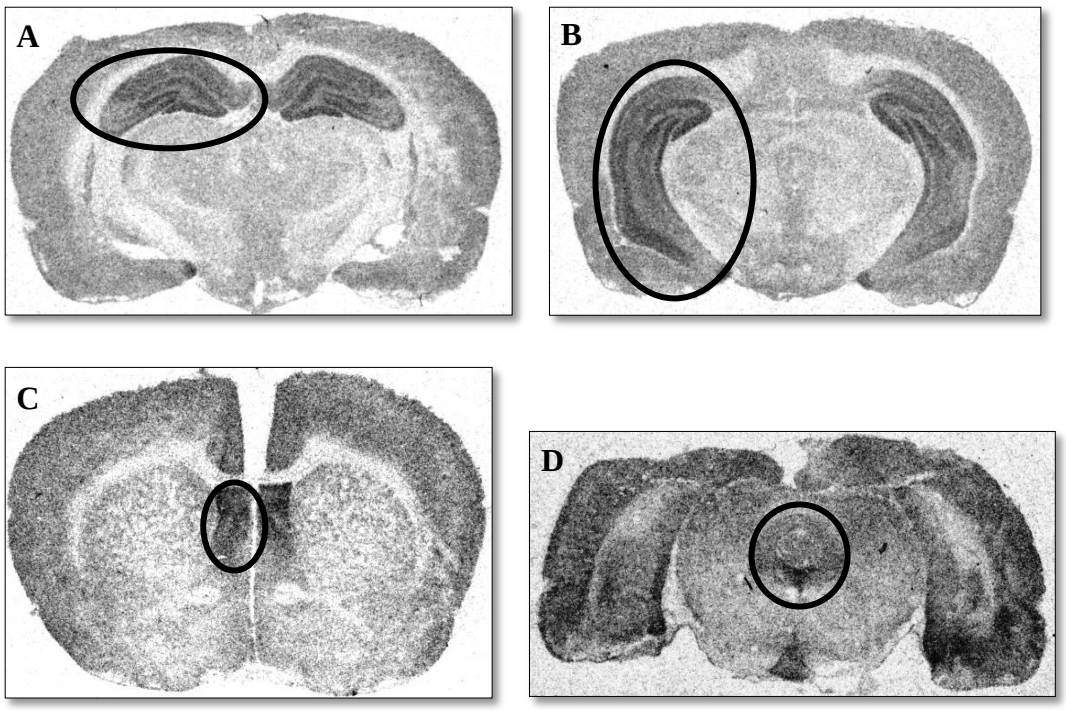

Figure 5.6 - Autoradiograms of Dex treated coronal male rat brain sections labelled with [^{3}H]WAY-100635 tritium ligand representing the dorsal hippocampus (A), ventral hippocampus(B), lateral septal nuclei (C) and raphe nuclei (D).

In the present study, 5HT-1A receptor binding was significantly increased in the dexamethasone (Dex) treated male rats compared with non treated control in the cingulate and prelimbic cortex, nucleus accumbens and dorsal hippocampal CA1,

CA2 and DG sub-regions. Binding was also significantly increased in Dex treated female rats versus non treated control in the cingulate and prelimbic cortex, septum, nucleus accumbens, ventromedial hypothalamus, dorsal hippocampal DG, ventral hippocampal - CA2 and CA3, and basolateral amygdala regions.

Univariate ANOVA analysis showed a significant main effect of treatment in the cingulate cortex ($F=24.\ 38$, $p<0.001$), prelimbic cortex ($F=14.\ 37$, $p<0.01$), lateral septum ($F=11.\ 23$, $p<0.01$) and nucleus accumbens ($F=21.\ 62$, $p<0.001$) regions. However, no significant main effect of sex or sex x treatment interaction was seen in these regions. There was significant increase in 5HT-1A receptor binding in both Dex treated males and females compared to controls in Cg (males (174%, $p<0.01$) and females (93%, $p<0.05$), PrL (males (67%, $p<0.05$) and females (48%, $p<0.05$)) and the Nacc (males (127%, $p<0.01$) and females (292%, $p<0.05$) regions. Only females, Dex treated vs controls, showed increase in the LSD region (40%, $p<0.05$) (Figure 5.7).

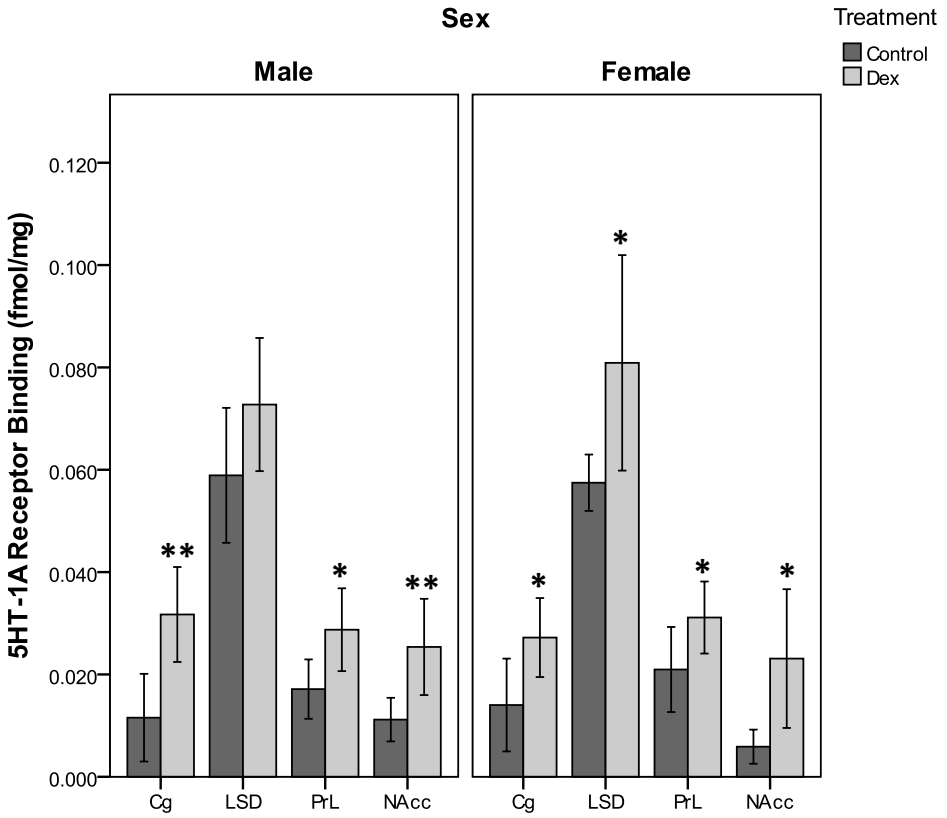

Figure 5.7 - 5HT-1A receptor binding in the cingulate (Cg) and prelimbic cortex (PrL), lateral dorsal septum (LSD) and nucleus accumbens (NAcc) in male and female rats in response to prenatal dexamethasone exposure.

Data expressed as mean values ± SEM in fmol/mg; n = 6 per group.

*p<0.05, **p<0.01 DEX versus control

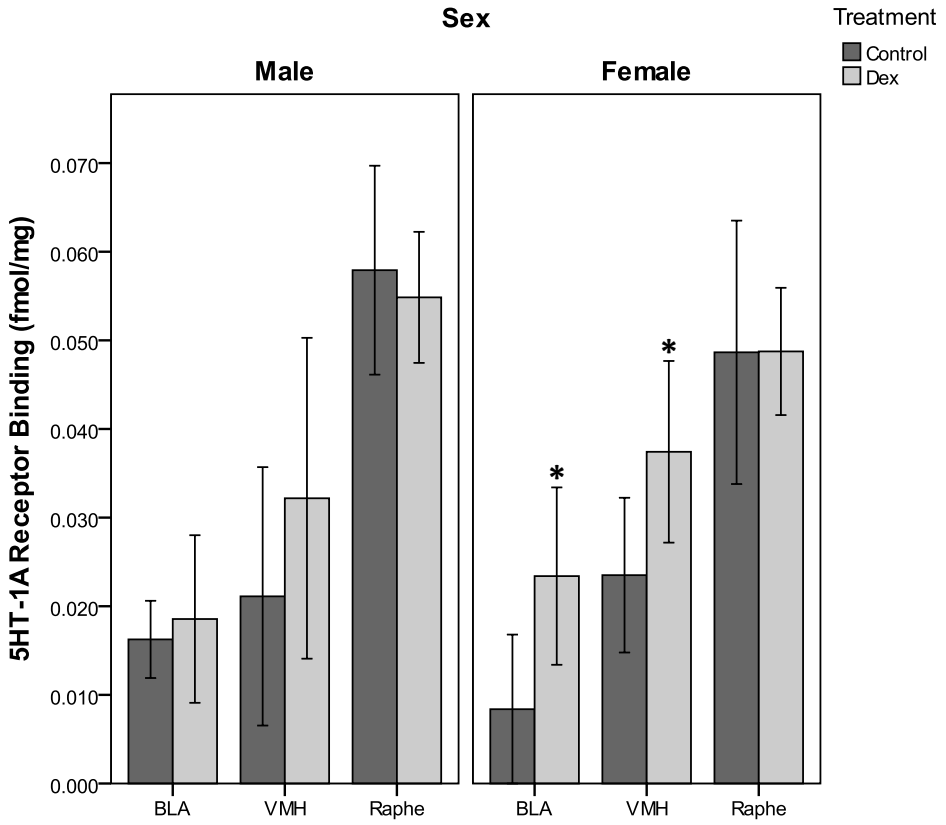

Figure 5.8 - 5HT-1A receptor binding in the basolateral amygdala (BLA), ventromedial hypothalamic nucleus (VMH) and raphe nuclei in male and female rats in response to prenatal dexamethasone exposure.

Data expressed as mean values ± SEM in fmol/mg; n = 6 per group.

*p<0.05 DEX versus control

A significant main treatment effect was observed in the basolateral amygdala ($F=7.09$, $p<0.05$) and ventro-medial hypothalamus ($F=5.71$, $p<0.05$). A significant increase was seen in the 5HT-1A receptor binding in DEX-treated females vs control in the VMH (59%, $p<0.05$) and BLA (179%, $p<0.05$) region. No treatment or sex effect was seen in the raphe nuclei (Figure 5.8). None of these regions showed difference in the sex x treatment interaction.

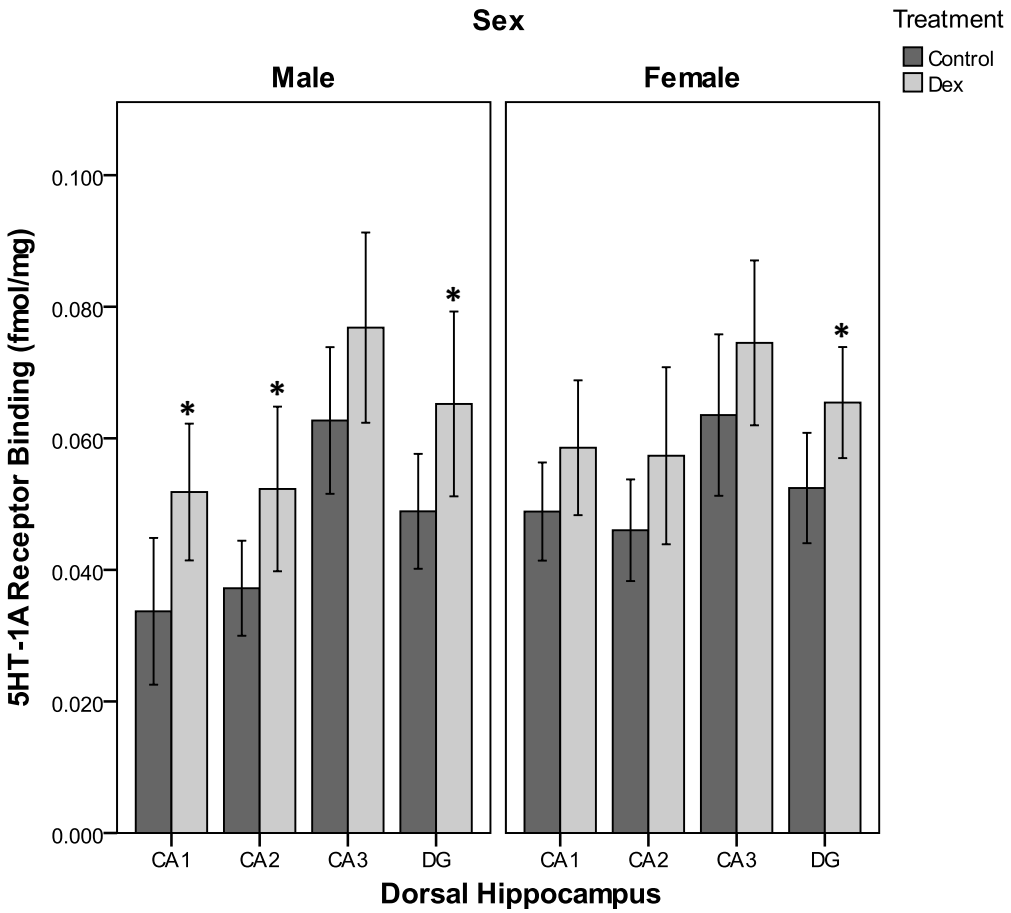

Figure 5.9 - Effect of prenatal dexamethasone exposure on the 5HT-1A receptor binding in the dorsal hippocampus region in male and female rats.

Data expressed as mean values ± SEM in fmol/mg; n = 6 per group.

*p<0.05 DEX versus control

The observed results in the dorsal hippocampal subregions show a significant main effect of sex in CA1 ($F=8.061$, $p<0.05$). In DEX-treated females, there was a significant increase in the 5HT-1A receptor binding in the DG region (24%, $p<0.05$ versus control). 5HT-1A receptor binding was increased in the dorsal hippocampal subregions of DEX treated males when compared with controls in CA1 (53%, $p<0.05$), CA2 (40%, $p<0.05$) and DG (33%, $p<0.05$) (Figure 5.9). There were no sex

x treatment interactions observed. Significant main effect of treatment was also seen in CA1 ($F=13.02$, $p<0.01$), CA2 ($F=10.25$, $p<0.01$), CA3 ($F=6.48$, $p<0.05$) and DG ($F=13.67$, $p<0.01$) dorsal hippocampal subregions (Table 5.2).

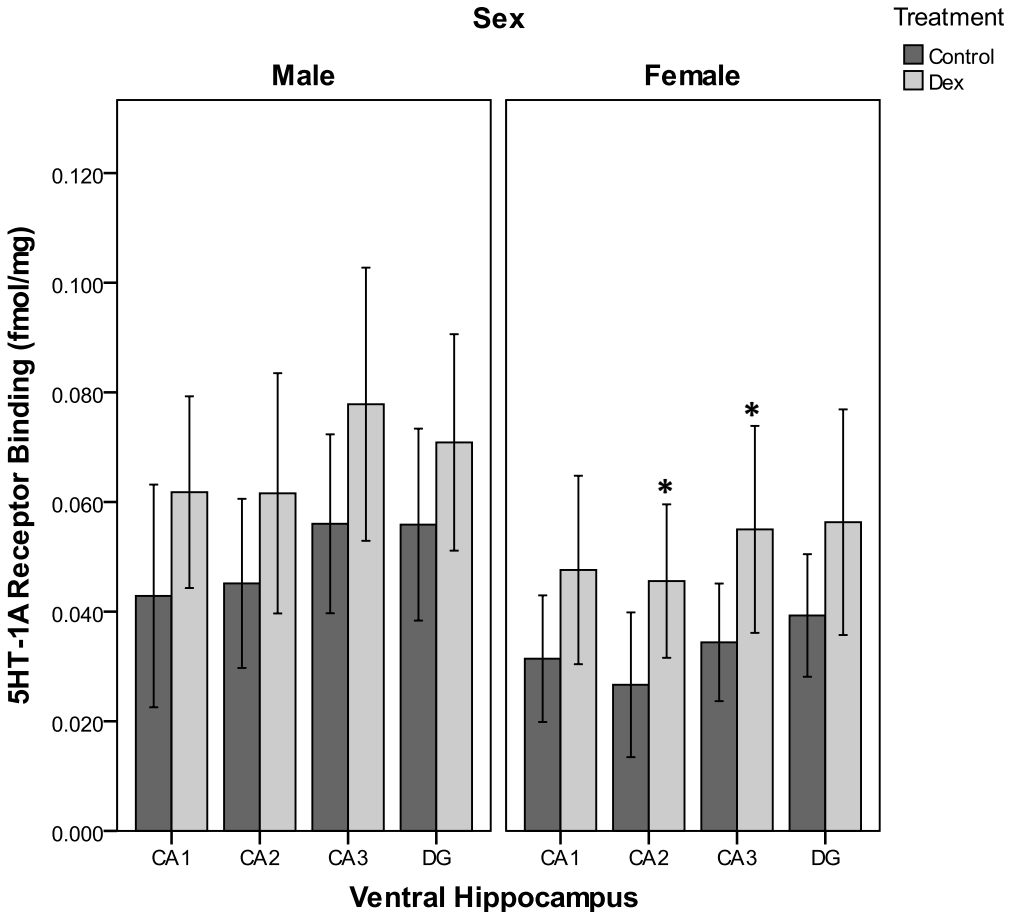

Figure 5.10 - Effect of prenatal dexamethasone exposure on the 5HT-1A receptor binding in the ventral hippocampal sub regions in male and female rats.

Data expressed as mean values ± SEM in fmol/mg; n = 6 per group.

*p<0.05 DEX versus control

The 5HT-1A receptor binding in DEX-treated males showed no significant changes whereas an increase in the binding was observed in females in the CA2 (70%, $p<0.05$) and CA3 (60%, $p<0.05$) sub regions, when compared with controls (Figure

5.10). There was a significant main effect of sex in the CA2 ($F=7.22$, $p<0.05$), CA3 ($F=9.61$, $p<0.01$) and DG ($F=5.14$, 0.05) area. Interaction between sex x treatment showed no significant differences. Main effect of treatment was seen in CA1 ($F=7.1$, $p<0.05$), CA2 ($F=7.58$, $p<0.05$), CA3 ($F=8.75$, $p<0.01$) and DG ($F=5.44$, $p<0.05$) (Table 5.2).

Table 5.2 - presents the statistical results of a univariate analysis of variance between sex and treatment in 5HT-1A receptor binding.

	Brain Region		**Source of variation**	**F_{value}** F(1,20)	**P_{value}**
5HT-1A Receptor binding	Cg		Sex	0.091	P > 0.05
			Treatment	24.383	P < 0.001 ***
			Sex x Treatment	1.069	P > 0.05
	LSD		Sex	0.364	P > 0.05
			Treatment	11.229	P < 0.01 **
			Sex x Treatment	0.744	P > 0.05
	PrL		Sex	1.173	P > 0.05
			Treatment	14.375	P < 0.01 **
			Sex x Treatment	0.064	P > 0.05
	BLA		Sex	0.218	P > 0.05
			Treatment	7.092	P < 0.05 *
			Sex x Treatment	3.825	P > 0.05
	NAcc		Sex	1.254	P > 0.05
			Treatment	21.625	P < 0.001 ***
			Sex x Treatment	0.199	P > 0.05
	VMH		Sex	0.534	P > 0.05
			Treatment	5.718	P < 0.05 *
			Sex x Treatment	0.074	P > 0.05
	Raphe		Sex	3.349	P > 0.05
			Treatment	0.125	P > 0.05
			Sex x Treatment	0.142	P > 0.05
	Dorsal Hippocampus	CA1	Sex	8.061	P < 0.05 *
			Treatment	13.020	P < 0.01 **
			Sex x Treatment	1.195	P > 0.05
		CA2	Sex	2.826	P > 0.05
			Treatment	10.255	P < 0.01 **
			Sex x Treatment	0.208	P > 0.05
		CA3	Sex	0.023	P > 0.05
			Treatment	6.488	P < 0.05 *
			Sex x Treatment	0.102	P > 0.05
		DG	Sex	0.224	P > 0.05
			Treatment	13.676	P < 0.01 **
			Sex x Treatment	0.177	P > 0.05
	Ventral Hippocampus	CA1	Sex	3.787	P > 0.05
			Treatment	7.100	P < 0.05 *
			Sex x Treatment	0.043	P > 0.05
		CA2	Sex	7.224	P < 0.05 *
			Treatment	7.584	P < 0.05 *
			Sex x Treatment	0.037	P > 0.05
		CA3	Sex	9.614	P < 0.01 **
			Treatment	8.748	P < 0.01 **
			Sex x Treatment	0.007	P > 0.05
		DG	Sex	5.142	P < 0.05 *
			Treatment	5.438	P < 0.05 *
			Sex x Treatment	0.022	P > 0.05

5.3 Discussion

5.3.1 Oxytocin receptor binding

The present study findings suggested that oxytocin receptor binding was affected by prenatal glucocorticoid treatment. It also indicated sex-dependent differences in the oxytocin receptor (OTR) binding amongst the control rats. Study found high intensity (24.05±1.21) of OTR binding in the central amygdaloid nucleus (CEM) in the females compared to male rats which supports the previously published findings (Liberzon and Young, 1997; Viero et al., 2010).

The secretion and release of oxytocin is oestrogen dependent, which may upregulate oxytocin in females and protects females against some of the factors affecting male social behaviours (Gimpl and Fahrenholz, 2001). This explains why major changes were observed in males compared to females in the present study.

Oxytocin receptors are widely distributed in the amygdala nuclei and have found to play a regulatory role in social recognition in the experimental mice (Ferguson et al., 2001; Winslow and Insel, 2002) and fear related behaviour in humans (Kirsch et al., 2005). Male rats exposed to prenatal stress showed significant increase in the levels of OTR mRNA expression in the central amygdala (Lee et al., 2007).

Considering the role of the amygdala in a range of social behaviours (Ferguson et al., 2001, Winslow and Insel, 2002) and its gender-specific functioning (Choleris et al., 2003), it is quite plausible to imply that behavioural changes observed in the prenatally stressed animals (Lee et al., 2007, Nagano et al., 2008) might be regulated by the altered oxytocinergic functioning in the amygdaloid nuclei. Also the fact that

development of the amygdaloid nuclei takes place earlier, as compared to the other brain regions, in the process of foetal maturation it is possible that prenatal dexamethasone treatment might affect the normal development and result into altered oxytocinergic systems (Lee et al., 2007).

The observed significant changes in the lateral septal nuclei region which is involved in the stress response (Ebner et al., 2000) supports the previous similar findings in the male rats (Lukas et al., 2010) and in female mice (Bales et al., 2011). Currently the specific functions of the oxytocin in the lateral septal nuclei remain unclear. However, there may be a possible link between the anti-anxiety action of the oxytocin (Gimpl and Fahrenholz, 2001) and the involvement of the septum in stress response (Ebner et al., 2000) which may suggest the altered mechanism of neurobiological response to stress induced by prenatal dexamethasone treatment.

The role of oxytocin as a regulator in social mating, partner preference and attachment which are mediated via nucleus accumbens (Ross et al., 2009a) which may suggest involvement of the oxytocin and dopamine pathways via mesolimbic reward circuit (Baskerville and Douglas, 2010). Therefore oxytocinergic system could play a role in the drug addiction and withdrawal behaviour acting via the nucleus accumbens.

Hippocampus being the crucial region involved in learning and memory processes (Woon et al., 2010) and also an area with abundant expression of glucocorticoid receptors (Liberzon and Young, 1997) is a likely target in prenatal dexamethasone exposure. Oxytocinergic system is also considered to be involved in the learning and memory regulation (Gimpl and Fahrenholz, 2001) which makes it a potential target to be affected by dexamethasone.

Previously decreased OTR expression in the dorsal hippocampus region was reported in postnatally stressed rats (Noonan et al., 1994). Glucocorticoids have shown an inhibitory effect on the hippocampus-mediated neurogenesis in the dentate gyrus (Woon et al., 2010) observed by lower levels of BDNF expression (Lemaire et al., 2000). Recently, Leuner et al. (2011) has reported that oxytocin supports hippocampal neurogenesis and neuronal proliferation in rats in the presence of glucocorticoids. Therefore, there is a need for further investigation to understand the role of oxytocin in hippocampal neurogenesis, which may become a potential therapeutic target.

Increased levels of OTR expression in the ventral hippocampal region in male rats exposed to chronic stress and corticosteroids have been observed (Liberzon and Young, 1997). Animal studies suggest an involvement of oxytocin in memory regulation (Popik et al., 1992) which suggests a role for the hippocampus. Green et al. (2011) observed reductions in the level of GC receptor protein in the hippocampus of male offspring exposed to unexpected maternal environmental stress, suggesting the link with the altered HPA axis negative feedback mechanism. Intracerebral infusion of oxytocin following stress exposure resulted in lower levels of GC receptor expression in the hippocampus in rats (Cohen et al., 2010).

Given the established interactivity of the central glucocorticoid and oxytocinergic systems (Gimpl and Fahrenholz, 2001), it is therefore reasonable to suggest that prenatal stress exposure inducing reductions in the cerebral GR expression might lead to elevated levels of the oxytocin receptors in the particular brain areas, in which the interaction between the GRs and the OTRs is intensive. However, this hypothesis needs to be expanded to include consideration of the gender-specific regulatory

mechanisms in the brain, such as the oestrogen receptors regulation of the OT-Rs in various areas.

In the brain, oestrogen has found to have pronounced effect on the OT receptor regulation. Literature suggests increased OTR's affinity (Caldwell et al., 1994) and increase in both density and area of OT binding (Coirini et al., 1991) in the VMH nucleus upon oestrogen administration. Oxytocin receptor mRNA expression in the VMH was also affected by oestrogen (Devidze et al., 2005). Therefore, the present findings of high levels of OTR binding in females in comparison to males could be due to the involvement of oestrogen. Observed reductions in the VMH - OTR binding in response to dexamethasone exposure prenatally might have similar mechanisms involved as seen in the altered VMH mediated sexual functioning and behaviour in rats exposed to early life stress (Lukas et al., 2010).

In the present study the high intensity (25.07±1.02) of OTR binding was seen in the subiculum transition (STR) area. The STR has not yet received much attention as its particular role in the CNS is not been recognised. However, Lowry (2002) suggests that subiculum may play an inhibitory role in the HPA axis reactivity to stress via the GABAergic neurones in the median raphe nucleus which project to hypothalamic nuclei (Lowry, 2002). Experimental studies in rats have shown high density of OTR binding in the subiculum area in the developing brain (Yoshimura et al., 1996, Barberis and Tribollet, 1996) suggesting subiculum area may play a regulatory role in the HPA axis function (O'Mara, 2005).

Interestingly, OTR binding in male rats was high in comparison to females which suggest gender variable may play a role. Melis et al (2009) reported oxytocin administration into the ventral subiculum resulted into increase in the levels of

dopamine in the nucleus accumbens and penile erection in male rats. The observed findings were concluded as ventral subiculum region might play a role in the reward and motivation behaviour via the mesolimbic dopaminergic reward pathway (Melis et al., 2009). Hence, it is justified to assume subiculum play a diverse role in the CNS, especially in relation to stress and reward pathway and needs further investigation.

5.3.2 5HT-1A receptor binding

The present study results highlight the observed altered brain regional sensitivity to prenatal dexamethasone exposure on the 5HT-1A receptor binding. However, there are only limited previous published data with which the present study can be compared (Fernandes et al., 1997; Andrew et al., 2003; Slotkin et al., 2006).

The interaction between the corticosteroids and the serotonergic system was first studied using autoradiography where increase in the 5HT-1A receptor binding was observed in the rats which underwent bilateral adrenalectomy after one week (Biegon et al., 1985).

Slotkin et al (2006) observed a significant increase (25% higher than the control) in the 5HT-1A receptor binding in brain subregions in rats exposed to dexamethasone during the GD 17-19 (0.05mg/kg dose). These Dex effects have been more pronounced in males (13%) than females (5%); they have been dose dependent (Slotkin et al., 2006). The present data are also in line with the above mentioned studies since as an increase in the 5HT-1A receptor binding was observed in response to dexamethasone administration (E16–E19, 0.5 mg/ml – present study).

Most of the previously published studies on the effects of glucocorticoids have focused on the hippocampal region considering its involvement and function in the CNS, e.g. (Fernandes et al., 1997; Andrew et al., 2003). The present study analysed several brain regions which also play an important part in emotional and cognitive behaviour and cognition. Previous studies have indicated that early stress exposure in the form of prolonged maternal separation did not influence the 5HT-1A receptor density or its mRNA expression in the dorsal raphe nucleus in rats (Arborelius et al., 2004). Whilst there is a discrepancy in the published data as to the direction of changes observed in the 5HT-1A receptor binding when analysed post mortem in depression (Stockmeier, 2003).

An increase in the 5HT-1A receptor density within the amygdala nuclei has been reported in adult rats exposed to brief maternal separation (Vicentic et al., 2006). The present study found an increase in the 5HT-1A receptor binding in the basolateral amygdala in female rats in response to prenatal dexamethasone administration. 5HT-1A receptor changes can play a role in disinhibiting amygdala responses to emotional stimuli (Fisher et al., 2006), potentially contributing to the pathological emotional behavior associated with mood disorders (Drevets & Raichle., 1992; Drevets, 2001).

The present study observed an abnormal increase in the cingulate and prelimbic cortex region, with no changes in the raphe nuclei in terms of the 5HT-1A receptor binding. These findings indicate an increase in the 5HT-1A receptor binding in the cortex could play a role in modulating the 5-HT release during stress (Haddjeri et al., 2000). It also seems that 5HT-1A receptor in raphe nuclei is not sensitive to circulating corticosteroids (Chalmers et al 1993).

A reduction in the 5HT-1A receptor sensitivity in the raphe nucleus in rats exposed to chronic dexamethasone treatment has been reported (Fairchild et al., 2003). In early deprived male Wistar rats 5HT-1A receptor binding has been significantly reduced in the cingulate and motor cortex and in the dorsal raphe nuclei (Leventopoulos et al., 2009). Significant decrease in 5HT-1A receptor binding *in vivo* was found in the raphe in depressed patients (Drevets et al., 2000, 2007). 5HT-1A receptor binding in raphe nuclei was found to reduce (Kassir et al 1998) and increase (Stockmeier et al 1998) in suicide victims compared to controls. However, there is a discrepancy in the findings of the 5HT-1A receptor binding in the cortex region where no difference between suicide and control samples has been found (Arranz et al 1994; Matsubara et al 1991; Stockmeier et al 1997) and in another study in suicide victim's increase was seen (Arango et al 1995).

Studies have shown changes in the glucocorticoid level affecting the function and the expression of the 5HT-1A receptors in the hippocampus (Rasmuson et al., 1998). Hippocampus is one of the most studied and affected region against the glucocorticoids for the obvious reason being, as high density of 5HT-1A receptors is expressed in the hippocampus (Zhong et al., 1995) and due to its important function in CNS. Hippocampus plays an important modulatory role in the affective and cognitive function (De Kloet et al., 1991). Enriched environment also results in the elevation of the hippocampal 5HT-1A receptor mRNA expression in rodents (Rasmuson et al., 1998). The highest number of glucocorticoid receptors has been found in the hippocampus region making it more sensitive to corticosteroid exposure (Harvey et al., 2003).

Substantial decrease has been observed in the 5HT-1A mRNA in the hippocampus in rats (Lopez et al., 1999). Leventopoulos et al (2009) reported a reduction in the motivation for reward and the suppression of 5HT-1A binding in anterior ventral hippocampal CA1, ventral dorsal raphe and cingulate cortex in early deprived Wistar rats. A significant reduction in the 5HT-1A receptor binding in the hippocampus was found in rats exposed to corticosteroids subcutaneously (Fernandes et al., 1997). The present study findings supports the earlier observations by Andrew et al. (2003) supports where the effect of dexamethasone on 5HT-1A receptors in guinea pigs exposed for 3 weeks during pregnancy period showed significant increase in the expression of the 5HT-1A receptors in the hippocampal CA1 and DG sub region (Andrew et al., 2003).

However, it is important to note that most of the previous studies were in response to postnatal manipulations and only a few studies reported effects of prenatal glucocorticoid exposure on the serotonergic system.

It is of relevance to point out that in the present study male rats showed a significant increase in the 5HT-1A receptor binding in the dorsal hippocampal subregions and not in the ventral hippocampal sub-regions. It could be due to the functional involvement of these regions: dorsal hippocampus is largely implicated in learning and memory whilst ventral hippocampus is involved in emotional processing (Bannerman et al., 2004).

Moreover, the hippocampal mediated learning and memory process is understood and animal studies have also shown that prenatal glucocorticoid therapy results into disruption of these processes (Emgard et al., 2007; Noorlander et al., 2007). Present

study findings might also suggest that 5HT-1A dysfunction can be the factor involved in the suppression of hippocampus-mediated function.

Considering the literature and present study findings along with general speculations the following possibilities could be the reason for 5HT-1A receptor alterations.

Possible mechanism underlying the increase in the 5HT-1A receptor binding could be due to the changes in the HPA axis programming, which occur at the time of dexamethasone treatment. It is reported that dexamethasone administration during the third week of gestation (14-19) results in the HPA axis alteration in the developing neuroendocrine system (Shoener et al., 2005, 2006). Dexamethasone exposure results in the maternal cortisol level reduction and dexamethasone preferentially binds to the GR receptors, which in turn alters the negative feedback regulation of 5HT-1A expression (Meijer & De Kloet, 1994; Welberg & Seckl, 2001). Therefore, this disruption may lead to the increase in the 5HT-1A receptor upregulation.

Another plausible explanation for the altered 5HT-1A receptor system links with membrane cholesterol. Studies have shown that increase in the cholesterol concentration could have a direct impact on the affinity of 5HT-1A receptors (Pucadyil & Chattopadyay, 2004). However, not many research studies have been carried out on the link between cholesterol and serotonin system under corticosteroid exposure but the increase in corticosterone is coupled to an increase in plasma cholesterol level (Barker, 1995). Further, it is known fact that 5HT-1A receptors are GPCRs that require cholesterol to function properly which also support the idea of cholesterol contributing to 5HT-1A receptor function (Peirce et al., 2002; Pucadyil & Chattopadyay, 2004). Further research should address the relationship between

corticosterone production and elevation in the brain cholesterol levels seen under stressful situation.

Interestingly, Slotkin et al (2006) have suggested that the effects of dexamethasone on the 5HT-1A receptor upregulation were observed regardless of serotonin levels. Ideally, an increase in 5HT-1A receptors due to the presynaptic serotonin release may lead to compensatory down-regulation of the postsynaptic receptors (Slotkin et al., 2006). However, this disconnection indicates an important mechanism underlying the effects of dexamethasone on serotonergic system activity.

Therefore, the present study findings and the previously reported literature suggest a myriad of interactions between the hypothalamic-pituitary adrenal axis and the serotonergic system (Chaouloff 1993), including findings that corticosteroids may attenuate post-synaptic 5HT-1A receptor function (Joels et al. 1991; Haleem 1992). This has led to the suggestion that the impairment in serotonergic neurotransmission seen in depression may be caused by the action of corticosteroids (Young et al. 1994) and it is plausible to assume possible mechanisms via which the observed 5HT-1A receptor changes might causally contribute to the behavioural changes seen in stress and depression.

5.4 Conclusions

Prenatal dexamethasone exposure induced significant alterations in the oxytocin receptor and serotonin 5HT-1A receptor binding in a number of brain regions, which regulate responses to stress, social cognition, emotional and sexual behaviour. These findings are particularly important as they emphasise the potential long-term responses to foetal overexposure to glucocorticoids and highlight the significance of a multifaceted interactions between the cerebral neuroendocrine system and neural adaptation to stress. It is tempting to extrapolate the present findings to human conditions supporting concern over widespread glucocorticoid therapy for pregnant women (Koenen et al., 2007). Considering the role of oxytocin and serotonin system in the central nervous system and in the mediation and regulation of social behaviour, the present findings suggest a need for further investigation of the roles of these neuromodulatory systems in the aetiology of stress related human disorders.

Chapter 6

Effects of dexamethasone treatment on cell proliferation and glial cell markers in neural stem cells

6.1 Introduction

As observed in the Chapters 3 and 4, postnatal early deprivation and prenatal dexamethasone exposure results in altered glial cell morphology in the form of reduced primary process length. Therefore, the present study has led to a further investigation of the *in vitro* effects of dexamethasone (Dex) exposure on neural stem cells (NSC) assessing glial cell markers.

As mentioned in Chapter 1, glucocorticoids (GCs) are important hormones which play a critical role in the intrauterine programming during the developmental period. GCs are involved in various functions – they control and regulate receptor expression, enzymes, various growth factors, ion channels and can act directly on genes and/or indirectly, affecting, other hormones (Buckingham, 2006). It is also well known that GCs are important for the adaptation to stress; still excess GC secretion can cause adverse effects on the central nervous system (de Kloet et al., 2005; McEwen, 2007).

Dex, a synthetic glucocorticoid, is used in many studies on GC effects both *in vivo* and *in vitro*. Previous studies have reported GC effects on neurodevelopment such as stunting of somatic growth, outright cerebral atrophy, and endocrine disruption (McEwen, 1992; Fuxe et al, 1994; Matthews, 2000; Maccari et al, 2003; Welberg and Seckl, 2001). Previous research studies have also reported Dex exposure leading to decreased cell proliferation (Noorlander et al., 2008), apoptosis (Yu et al., 2010), reduced cell size (Kreider et al., 2006) and ability to retard neuronal migration (Fukumoto et al., 2009). GC administration in rodents affects hippocampus, hypothalamus, cortex and cerebellum by an increased programmed cell death (Duksal et al., 2009; Zhang et al., 2002; Li et al., 2010). Experimental studies also

suggest that stress and glucocorticoids are among the potent inhibitors of neurogenesis in the adult dentate gyrus (Czeh et al, 2002; Mirescu & Gould, 2006). A decrease in the rate of neurogenesis in the adult hippocampal dentate gyrus has shown to account for the hippocampal volume loss (Henn & Vollmayr, 2004; Czeh & Lucassen, 2007).

It has been shown that the production of various cytokines is differentially affected by GCs, both *in vivo* and *in vitro* (Barnes, 1996; Brattsand and Linden, 1996). Cytokines, known as immunomodulating agents, mediate the neural components of the host response to infection and are involved in the neurodegenerative processes resulting from brain trauma, ischaemia and chronic neurodegenerative diseases. However, they are also involved in CNS repair and recovery (Molina-Holgado and Molina-Holgado, 2010). Interleukin- 1β (IL-1β), an important pro-inflammatory cytokine, is involved in the development of brain inflammatory responses during infection, trauma and neurodegenerative processes (Hopkins and Rothwell, 1995; Dinarello, 1998). IL-1β plays a key role in a number of brain pathologies by inducing astrogliosis and promoting neuronal cell death directly or indirectly via the production of toxic mediators such as inflammatory cytokines, reactive oxygen intermediates and nitric oxide (Sparacio et al. 1992; Lee et al. 1995; Rostworowski et al. 1997; Pearson et al. 1999). However, brain injury is associated with an enhanced release of glucocorticoids (due to HPA axis activation) which inhibits IL-1β production and transcription (Berkenbosch et al. 1987; Lee et al., 1988; Linden and Brattsand, 1994).

Astroglial cells, known to be regulated by glucocorticoids, play a pivotal role in brain inflammation in response to trauma, ischemia and infection, and are the most

important cell type involved in the tissue repair in central nervous system (Ridet et al. 1997; Newman, 2003). As it has been reviewed in the general introduction, impaired astroglial function is reported in many medical conditions (Seifert et al., 2006) such as loss and morphological alteration in depressive disorders (Bowley et al., 2002; Rajkowska and Miguel-Hidalgo, 2007) and astroglial overactivation and hypertrophy in Alzheimer's disease (Mrak and Griffin, 2005). Evidence also suggests that glucocorticoids produced during an inflammatory response may directly modulate astrocyte functions and inhibit astrocyte proliferation (Crossin et al. 1997; Huang and O'Banion 1998; Pousset et al. 1999). A recent study also demonstrates the importance of astrocytic functioning and IL-1 signalling, being critical for the hippocampal-dependent learning and memory processes (Ben Menachem-Zidon et al., 2010).

Therefore, the purpose of the present study was first to study the effects of the synthetic glucocorticoid Dex on the expression of the glial marker, glial acidic fibrillary protein (GFAP), and secondly, the proliferation, differentiation and fate specification of NSC in the presence of the Dex.

6.1.1 Aims and Hypotheses

As dexamethasone affects the volume of hippocampus in a long-term, and as adult hippocampal plasticity implicates neurogenesis, the present study evaluated the effects of dexamethasone on the proliferation and differentiation of neural stem cells. The specific research questions were as follows:

Does *in vitro* dexamethasone treatment affect the cell survival?

Does dexamethasone treatment result in a reduction in the proliferation and glial cell differentiation in NSC?

6.2 Results

6.2.1 LDH cytoxicity assay

LDH analysis indicated no significant treatment effect in wild type (WT) [F (3, 8) = 3.030, p>0.05] mice. (ANOVA with Post Hoc - Tukey test, n=4 per group).

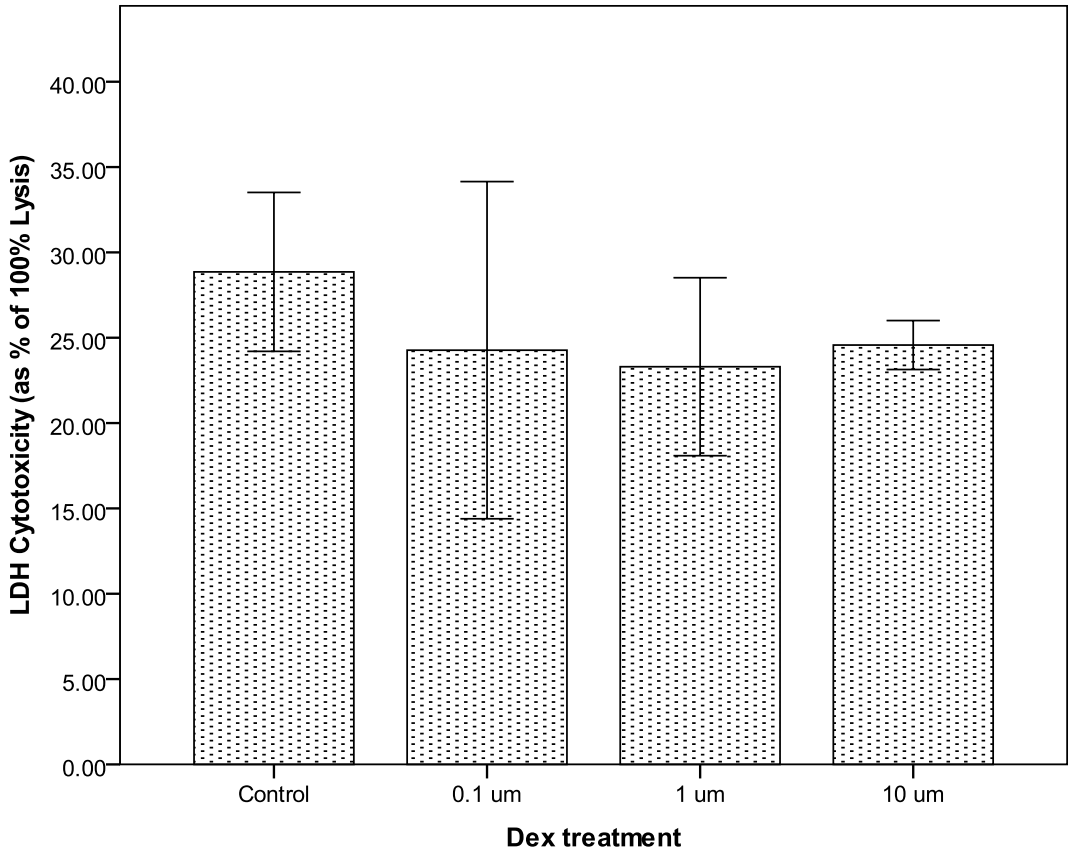

Figure 6.1 – LDH assay reveals no cell death under Dex exposure (0.1, 1 and 10 µM) in NSC cultures (t=24h) from WT mice.

(Data are presented as mean±SEM, n=4 per group)

6.2.2 Proliferation

Dexamethasone treatment induces decrease in the proliferation of neural stem cells in WT [F (3, 12) = 4.180, P<0.05] mice assessed by total cell numbers in a dose dependent manner (Figure 6.2). However, Control x 10µM dose showed high significant difference in both WT (P<0.05) mice. (ANOVA with Post Hoc - Tukey test, n=4 per group).

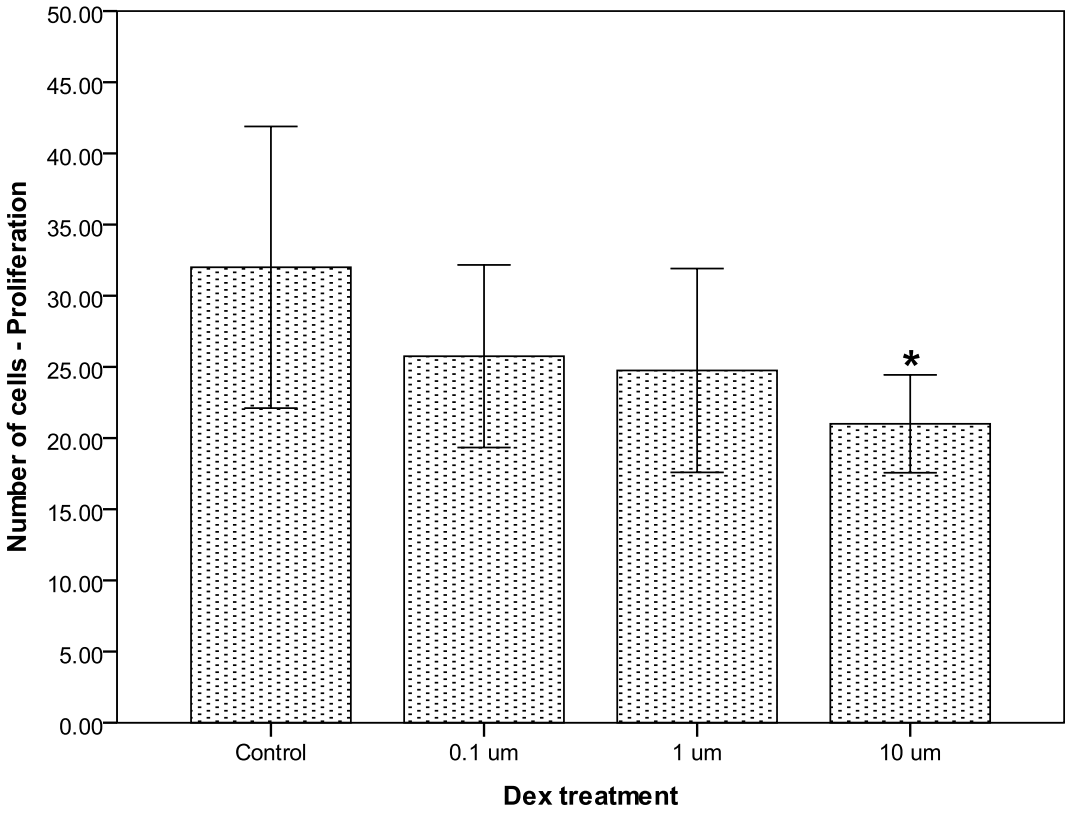

Figure 6.2 – Dexamethsone treatment showed a decrease in the proliferation (t=48h) rate of NSC from WT mice.

(Data presented as mean ±SEM, n=4 per group) (*p<0.05, **p<0.01)

6.2.3 Differentiation

Dexamethasone treatment results into decrease in the differentiation of GFAP positive cells in WT [F (3, 12) = 5.458, P<0.05] mice assessed by GFAP positive cell numbers. However, Control x 10µM dose showed high significant difference in WT (P<0.05). (ANOVA with Post Hoc - Tukey test, n=4 per group).

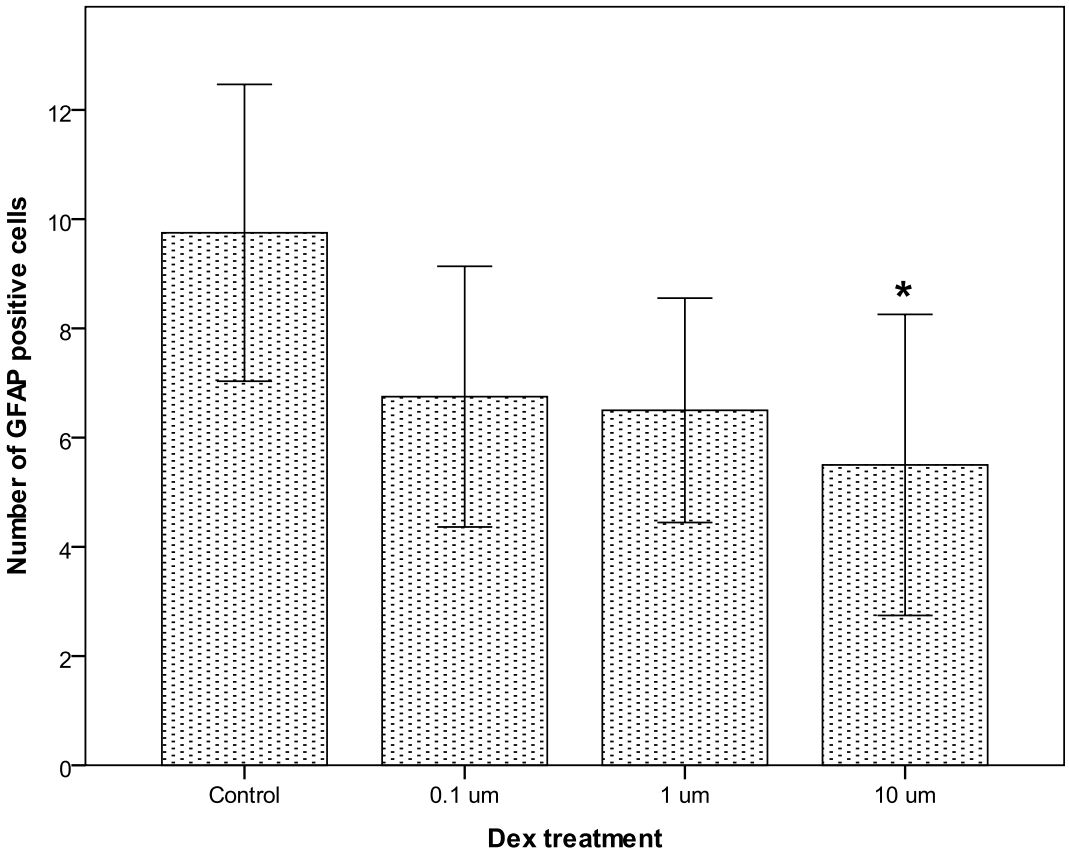

Figure 6.3 – Decrease in the number of GFAP positive cells was seen in WT mice with dexamethasone exposure (t=48h).

(Data are presented as mean ±SEM, n=4 per group). *p<0.05 vs control.

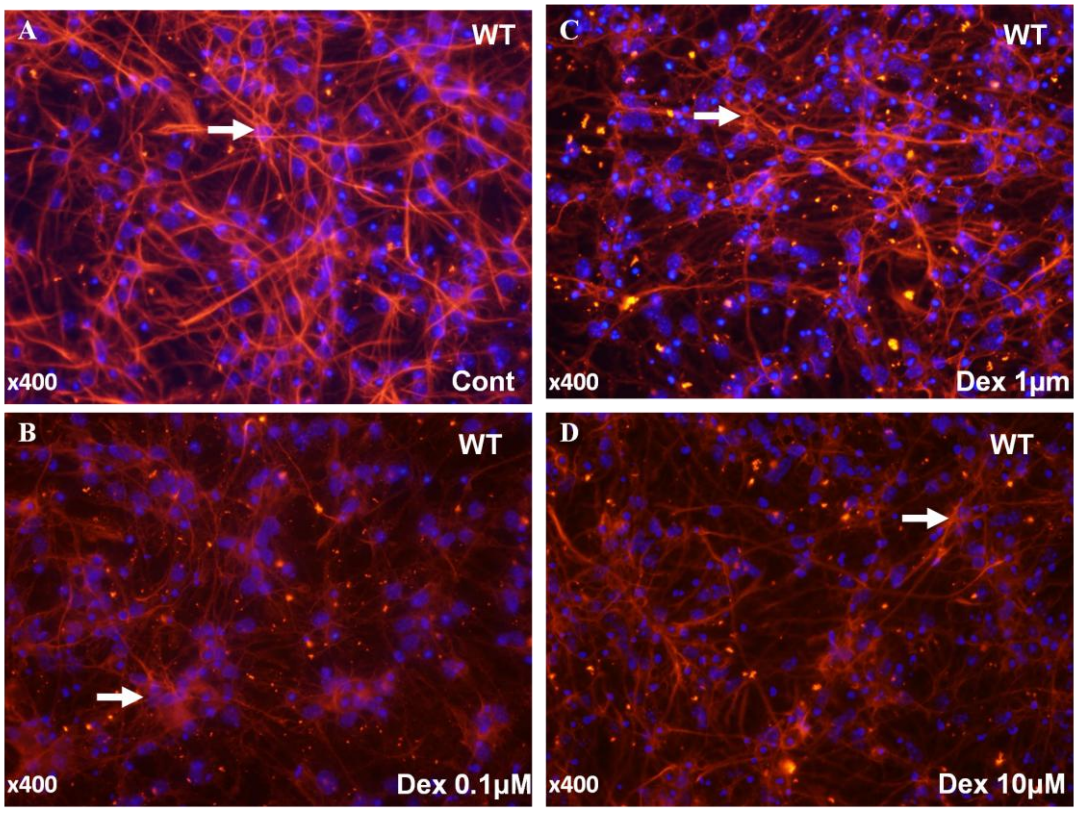

Figure 6.4 - The micrographs represent the role of dexamethasone in the differentiation of the NSC from WT mice. Dexamethasone induces a decrease in the differentiation of GFAP positive cells (indicated by white arrow) in WT mice in different concentration dependent manner.

Panels show phase contrast images of untreated NSC (Cont – A) and NSC exposed (t=48h) to increasing concentrations of Dex (0.1µM – B; 1µM – C; 10µM – D).

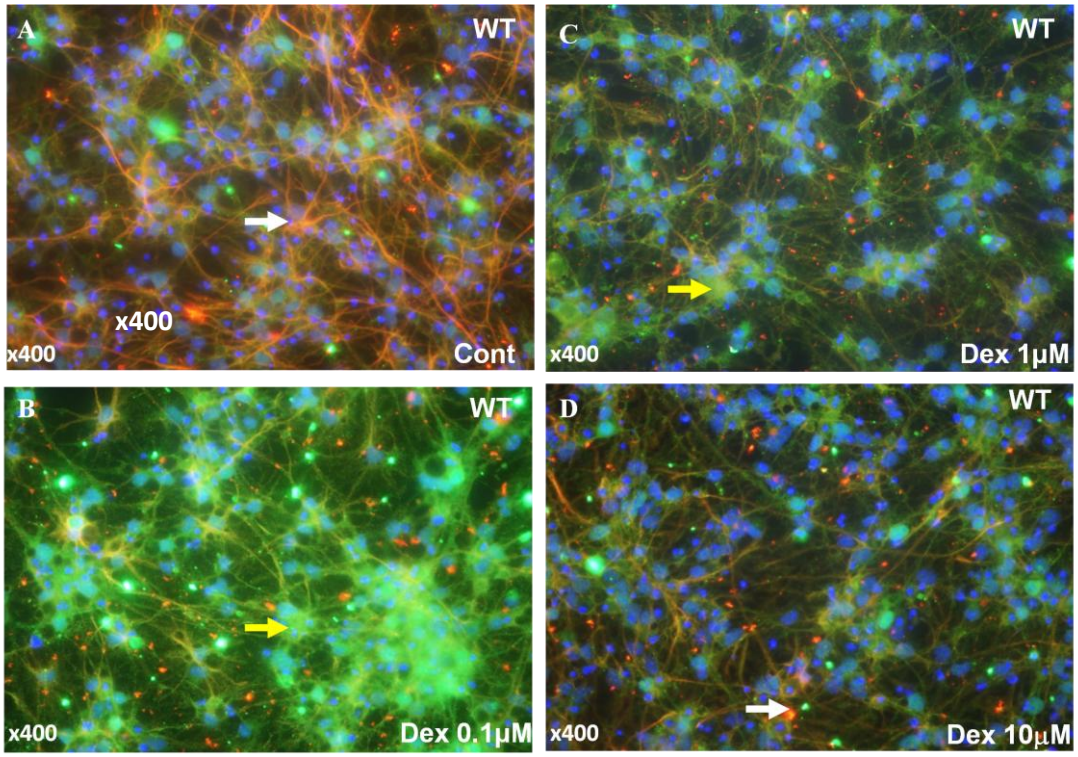

Figure 6.5 – The micrographs represent the effect of Dex on GFAP (red) (indicated by white arrow) and S100 (green) (indicated by yellow arrow) positive cell differentiation in WT mice. Dex induces a decrease in NSC proliferation in different concentration dependent manner.

Panels show phase contrast images of untreated NSC (Cont – A) and NSC exposed (t=48h) to increasing concentrations of Dex (0.1μM – B; 1μM – C; 10μM – D).

6.3 Discussion

The astroglial changes observed in the rats exposed to prenatal dexamethasone (Dex) (E16-19) (refer Chapter 4) and underwent early deprivation (P1-14) (refer Chapter 3), in patients suffering from depression (Bowley et al., 2002), in relation to stress (Leventopoulos et al., 2007, Czeh et al., 2010) and its involvement in brain plasticity (Jauregui-Huerta et al., 2010) supports to the further analysis of *in vitro* effects of Dex on neural stem cells (NSC). Therefore, the present study aimed to identify the effects of Dex on the GFAP positive astroglial cell proliferation, differentiation and its cytotoxicity in NSC.

6.3.1 Impact of dexamethasone on astroglial cell

The present study findings indicate that transient exposure of NSC cultures to dexamethasone limits proliferation of neural progenitors. Dexamethasone also decreases the GFAP positive cell differentiation in WT mice.

As described previously (Chapters 1 and 4), preclinical and clinical studies have found damaging effects of dexamethasone exposure which leads to disruption of HPA axis (Seckl, 2004), reduction in glial cell proliferation both *in vitro* (Crossin et al., 1997) and *in vivo* (Wennstrom et al., 2006). Neurodevelopmental delay and cerebral palsy along with differences in morphology and structure of the offspring's brain treated with dexamethasone have also been reported (Halliday, 2002; Murphy et al., 2001; Choi et al., 2004).

On the other hand, clinical studies have shown Dex to induce protective effects against the damaging effects resulted from the CNS inflammatory response and lumbar cerebrospinal fluid (CSF) pressure resulting from bacterial meningitis (Lebel et al., 1988; Odio et al., 1990). Dex therapy is also found to decrease the

concentrations of IL-1β and TNFα in CSF of patients with bacterial meningitis (Mustafa et al., 1989). Hence these findings indicate Dex plays a neuroprotective role in the bacterial meningitis by reducing the IL-1β levels in the CNS.

The present study findings suggest that Dex affects the proliferation of NSCs. In support of these findings, Bose et al. (2010) have also found that Dex (1μM, t= 48 h) leads to decrease in the proliferation of NSC with no changes seen in the cell viability or differentiation. Sundberg et al. (2006) also reported a decrease in the NSC proliferation in rats exposed to Dex (100μg/kg, t=72 h) during the prenatal period (E14). In terms of cellular mechanisms, the decrease in NSC number induced by Dex that was observed in the present study was not associated with an increase in cell death which evidently indicated an alteration in the proliferation and or differentiation rate.

Astrocytes play a major role in restoring homeostasis to the damaged brain and IL-1β regulates multiple astrocytic responses after injury (Basu et al., 2004; John et al., 2005). It is also know that astrogliosis and astroglia function play a crucial role in the pathogenesis of many neurological disorders (Mucke & Eddleston, 1993; Basu et al., 2002). In inflammation an increase in the size and number of astroglia cells is observed in the guinea pig ileum (Gabella, 1984; Bradley; 1997).

6.3.2 Dexamethasone cytotoxicity in brain cells

The current study findings suggest that the presence or absence of Dex did not affect the NSC survival and had no toxic effects on NSC in the presence of dexamethasone confirmed by LDH cytotoxicity test.

6.3.3 Effect of dexamethasone on neuronal and glial cells *in vitro*

Glucocorticoid hormone, in order to mediate its function, acts via its receptors that have been identified in vitro in both neurones and astroglia (Chou et al.1991; Bohn et al. 1994). Studies have also shown that in vitro, glucocorticoids suppress the production and activities of proinflammatory cytokines on cells of the monocyte-macrophage lineage and astrocytoma cell lines (Kern et al. 1988; Lew et al. 1988; Nishida et al. 1989). It is also known that cytokines in association with the activated glia may affect or regulate neuronal function (Srinivasan et al., 2004). Hence the present study aimed to identify the effect of dexamethasone on the neuronal and astroglial cell in a concentration dependent manner. No changes were seen in the beta III positive neuronal cells in WT mice.

6.4 Conclusions

Transient exposure of neural stem cell culture to Dex limits proliferation of neural progenitors, which is manifested by an decrease of NSC differentiation rate, evaluated by cell numbers in a concentration dependent manner. Dex treatment also affects the GFAP differentiation in NSC from WT mice.

Cytotoxicity tests (LDH assay) suggest that Dex does not affect NSC survival and has no toxic effects on NSC.

The present study provides evidence, in support to Chapter 4, for the effects induced by Dex in NSCs and supports the idea that prenatal exposure of GC may induce long-term effects of relevance to neurodevelopmental and/or neurodegenerative disorders.

Chapter 7

General Discussion

The aim of the present study was to investigate the long term effects of early life stress, during the prenatal and postnatal period, with a main focus on the brain remodelling in relation to the regional volume and astroglial morphology in adult rats.

7.1 Highlights of the present findings

Study 1 – Long term effects of ED on the hippocampal volume and astroglial cell morphology (Chapter 3)

This study was carried out in male adult Wistar (stress normo-responsive) and Lewis (stress low-responsive) rats which underwent postnatal manipulations in the form of ED and EH, using the model of early life deprivation developed at the animal facility of the Swiss Federal Institute of Technology, Schwerzenbach, Switzerland. The results obtained in this study indicate that postnatal ED resulted in a region specific hippocampal volume loss (by 12.5%) in Wistar's with no changes in Lewis rats. Significant differences were not seen in the basolateral amygdala and nucleus accumbens in both Wistar and Lewis rats. ED in Wistar rats also affected GFAP-positive astroglial cell morphology in the dorsal hippocampus – a significant reduction in the primary process length and number was observed. ED did not affect the total brain volume measured by using the pQCT scanning technique.

Study 2 - Prenatal dexamethasone exposure affects hippocampal volume and astroglia morphology (Chapter 4)

In this study, male adult Sprague Dawley rats exposed prenatally to dexamethasone (glucocorticoid) were studied as an animal model of stress-related prenatal manipulations established at the Faculty of Medicine, Imperial College London, UK.

Moderate yet significant volume loss (by 8%) and reduction in the astroglial primary process length was observed with no changes in the total and astroglial cell number in the hippocampus. No treatment effects in the total brain volume were seen as measured by means of pQCT.

Study 3 – Altered oxytocin and 5-HT1A serotonin receptor system in male and female adult rats exposed prenatally to dexamethasone (Chapter 5)

It was an extension of study 2 with an aim to investigate effects of prenatal exposure to dexamethasone on the selected neuro-regulatory processes implicated in responses to stress and glucocorticoids. The findings indicate a decrease in the oxytocin receptor binding in the Nacc, ventral hippocampal CA1, CA2 and CA3 and in the PVN region in males. In females, a decrease was seen in the LSD, ventral CA3 and VMH regions. An increase in the oxytocin receptor binding was observed in dorsal CA2 and PVN region in females.

5-HT1A receptor binding was found to be increased in Cg, PrL, Nacc, dorsal – CA1, CA2 and DG in males. In females, increase was observed in the Cg, PrL, Nacc, LSD, ventral - CA2 and CA3, dorsal DG, BLA and VMH regions.

Study 4 – Dexamethasone affects proliferation and differentiation of the neural stem cells in a concentration dependent manner (Chapter 6)

The aim of this study was to investigate the effects of dexamethasone on the expression of the glial marker GFAP in the NSC proliferation and differentiation. Cortex-derived NSCs of C57BL/6J - WT mice were exposed to dexamethasone at several concentrations (0.1 µM, 1 µM and 10µM). Cytotoxicity assay did not show dexamethasone dependent cell death. A decrease in the NSC proliferation rate was

observed in response to increasing Dex concentration. The study also found a Dex-dependent reduction in the GFAP positive cell differentiation.

7.2 Present findings on the background of existing research

The outcomes of the present study 1on the postnatal early deprivation, and study 2 on the prenatal dexamethasone exposure, indicate that both prenatal and early postnatal environmental manipulations lead to a region-selective hippocampal volume loss. The present hippocampal volume reductions corroborate the observations reported in the clinical studies in human patients suffering from depression (Viderbech and Ravnkilde, 2004), PTSD (Apfel et al., 2011), following sexual abuse (Sapolsky, 2000) and childhood neglect (Teicher et al, 2004), and in the experimental studies on chronic psychosocial stress in the tree shrew (Czeh et al., 2006), early life deprivation in rats (Lehmann et al., 2002; Opacka-Juffry *et al*, 2008) and corticosteroid exposure in primates (Uno et al., 1990; 1994).

Hippocampus is the region involved in, the processes of learning and memory and the regulation of the HPA axis functioning. It is sensitive to stress and stress hormones; its cells express abundant glucocorticoid and mineralocorticoid receptors (de Kloet et al., 2005) and adult hippocampus shows neuronal cell proliferation (Gould *et al*, 1997; Czeh *et al*, 2002; Mirescu & Gould, 2006). The present hippocampal volume losses, apparently modest, in the range of 8% - 10% are similar in their extent to the changes reported in humans (Cambell *et al*, 2006; Videbech & Ravnkilde, 2004). They may have behavioural implications in the form of

hippocampus-dependent memory malfunctioning (Woon et al., 2010). However, the limitation of the present study is a lack of direct behavioural observations.

Study 3 also found altered oxytocin and 5-HT1A receptor binding in the hippocampus region. It is known that the hippocampus, apart from the above mentioned abundant expression of glucocorticoid and mineralocorticoid receptors, also has a high density of 5-HT1A receptors (Zhong et al., 1995).

The central 5-HT1A receptor undergoes alterations in response to stress (Lopez et al., 1999; Arborelius et al., 2004) and depression (Drevets et al., 2007). The HPA axis effects on 5-HT1A receptor binding has been suggested in the hippocampus region (Lucki et al., 1998).

Dex administration during the prenatal period (G14-19) results in the HPA axis alteration in the developing neuroendocrine system (Shoener et al., 2005, 2006). As mentioned earlier, hippocampus plays a role in the CNS, is involved in the HPA axis negative feedback mechanism and the rich presence of 5-HT1A receptors, makes it an important brain region and likely target in stress related disorders.

ED has shown to affect the HPA axis functioning with attenuated ACTH and corticosterone stress responses in rats (Nunez et al., 1996; Pryce et al., 2001; Lehmann et al., 2002). Elevated glucocorticoid levels are being observed with ED in rats (Levine et al., 1988; Dent et al., 2000; Oomen et al., 2009). Therefore it is plausible to assume that there is a common factor, namely glucocorticoid overexposure, in both early deprivation (study 1) and prenatal Dex treatment (study 2) animal models.

Excessive exposure to GCs can underly both the present increase in the 5-HT1A receptor binding (study 3) and the morphological changes seen in the hippocampus (studies 1 and 2), which result from the ED and/or dexamethasone treatment. It is also important to note that, in studies 1 and 2, no significant treatment effects on the total brain volume and non-hippocampal regions were observed, which suggests an involvement of the hippocampal mediated vulnerability (Heim and Nemeroff, 2001; McEwen, 2003).

Oxytocin has been found to increase the astroglial proliferation rate in the cultured cortical and hypothalamic cells (Lucas and Salm, 1995) and astroglial cells express both oxytocin and 5-HT receptors in the brain (Whitaker et al., 1993). GFAP expression is found to fluctuate during the pregnancy and lactation period where oxytocin plays an important role (Gomora-arrati et al., 2010). The present observations suggest that Dex exposure alters the GFAP positive glial cell - morphology (study 2), proliferation and differentiation (study 4) and the OTR and 5-HT1A receptor alterations (study 3). The above supports the view of a common mechanism, which underpins the findings of these three studies, although it needs to be further investigated in future studies.

An important and novel finding of the present study was a reduction in the primary process length of GFAP positive astroglial cells in the hippocampus, observed in both the early life stress and prenatal exposure to glucocorticoids. A previous ED study in stress hyper-responsive Fischer rats has shown a reduction in the astroglial density (Leventopoulos et al., 2007). Both preclinical and clinical studies have shown changes in glial cells in terms of proliferation (Crossin et al., 1997;

Wennstrom et al., 2006), reduced cell numbers (Rajkowskal., 2000; Bowley et al., 2002) and expression (Nichols et al., 1990a,b; Tsuneishi et al., 1991).

It is tempting to interprete the ED and prenatal Dex effects on astroglia as a manifestation of some long-term reductions in the brain plasticity. Also the observed changes in the astroglial morphology in the form of primary process length reduction may underpin the observed hippocampal volume loss. It is possible to assume that glial responses could also be the reasons for the volumetric losses reported in patients suffering from depressive disorders (Miguel-Hidalgo et al 2000; Müller et al 2001; Bowley et al., 2002; Rajkowska and Miguel-Hidalgo, 2007).

7.3 Novelty of the present study

The present thesis has provided some novel and interesting developments and findings in terms of both methodolgy and data. It has delivered new knowledge and indicated further directions in the understanding of the mechanisms involved in long term effects of early life stress.

The present study was the first one to compare the experimental ED - Wistar (stress normo-responsive) and Lewis (stress non-responsive) rats in terms of total brain and regional volumetry and astroglia morphology. Previous ED studies have analysed only one rat strain at the time (Pryce et al., 2001a; Leventopoulos et al., 2007; Leventopoulos et al., 2009; Oomen et al., 2009). Wistar and Lewis rat strains have been compared for behaviour in the Morris water maze (Teunissen et al., 2001), Y maze learning task (Sida et al., 2003), open field test (Rex et al., 1996) and forced

swimming test (Armario et al., 1995). However the present study has compared ED effects on these rat strains at the anatomical and cellular level for the first time.

Another novel approach of the present study was the comparison between NH, EH and ED groups. Previous studies have compared these three groups only in terms of behaviour (Pryce et al., 2001a; Rüedi-Bettschen et al., 2005) and maternal care (Pryce et al., 2001b). Studies by Leventopulos et al. (2007, 2009) have measured the GFAP density and serotonin receptor binding in NH and ED groups only. The inclusion of the EH group in the present study is important as it controls for the handling that takes place during the caging, cleaning and breeding (Levine et al., 2001; Pryce et al., 2003).

The use of the sought after stereological quantitative analysis makes the present work unbiased as it applies systematic random sampling estimation of the regional brain volume, cell number and length (Schmitz and Hof, 2005). To my knowledge, the present study is the first one to apply stereology to the analysis of anatomical brain changes caused by prenatal exposure to dexamethasone in adult rats.

In order to assess the astroglial morphology, the present study applied the Neurolucida system – a stereology technique to measure the primary process length and number. The previous studies have reported astroglial density and number responses to ED (Leventopoulos et al., 2007) but the present study is the first one to measure the length of the primary processes using stereology.

In terms of the methodology, peripheral quantitative computed tomography was employed in a novel approach to measure total brain volume post-mortem in a high-precision manner, which is important as overall brain size itself is a variable in

neuroanatomy and neurobiology research. In this respect, the present study shows that pQCT can be used as a tool for soft tissue (brain) and not only hard (bone) tissue volume analysis. This approach is of relevance to volumetric studies on neuroplasticity where regional volumes need to be scaled to the total brain volume; thanks to this the present study could estimate the relative hippocampal volume, similarly to human studies by means of MRI.

Following on the observations of the consequences of prenatal dexamethasone exposure in rats (Study 2), the present study also evaluated *in vitro* effects of dexamethasone in neural stem cells (Study 4).

7.4 Limitations

The present study suffers from some limitations mainly due to the time restrictions. Firstly, there is a lack of behavioural studies with which the observed post-mortem changes could have been correlated. Although there are published studies in ED rats, which show altered learning and memory based tasks (Pryce at el., 2003; Rüedi-Betchen et al., 2005) which correlate with the present findings concerning the hippocampus, it would have been ideal to match the behaviour with the post mortem data of the same experimental animals. Behavioural tests such as Morris water maze, forced swim and US preexposure tests could have been performed.

Secondly, no neuronal analysis was completed in the present study. Previous studies have shown a decrease in the neuronal number, neurogenesis and dendritic atrophy in relation to the effects of stress and depression (McEwen, 2001, 2005; Duman, 2002, 2004; Fuchs et al., 2004). Although it had been planned to assess neurone-astroglia interactions, that was abandoned for technical reasons and the present project

focused mainly on the astroglial cells, as less studied in the litearture. However, it is interesting to know if ED and prenatal Dex exposure affect neurones along with astroglial cells. Hence stereological analysis of neuronal numbers and dendritic length should have been done.

Lastly, a small sample size was analysed in the present study. Considering the ethical regulations and with the attempt to reduce use of animals in research, six animals per treatment per experiment were used. Previously published studies and the in-house experience were also taken into account while designing the experiment. However, despite the small sample size the present study findings still indicated significant effects.

7.5 Future directions

The present thesis findings suggest the need for further experiments which could add more details and new understanding to the current observations.

The present study attempted to analyse neurone-glia interactions using double layer – immunohistochemistry; however, the experiments were unsuccessful. It is important to understand the colocalisation as astroglial impoverishment was observed in the current study. Both neurones and glial cells communicate with each other and control various important processes of brain development and homeostasis (Bhat, 2003; Gomes et al., 1999; Lim & Alvarez-Buylla 1999). Also glial cells are important for neuronal cell surviaval as they provide energy substrates for neurones (Dringen et al., 1993; Cohen et al 1999). Therefore it is pivotal to know about the neurone-glia interaction and hence there is a need for further study.

There is also a need to analyse the effects of ED and prenatal Dex treatment on the glial cell genesis (gliogenesis). The present study observed loss of glial cell number (ED) and primary process length reduction (ED and Dex). It will be interesting to know that if these animal models affect the formation of new glial cells. This can be evaluated by immunohistochemistry technique by staining 5-bromo 2-deoxyuridine (BrdU) and GFAP positive astroglia colocalisation (Gould et al., 1997; Lemaire et al., 2000).

The observed strain differences in the study 1 suggests an involvement of genetic mechanisms (McEven et al., 2012) as exposure to stress in early life results in 'environmental programming' (Levine, 1994; Pryce et al., 2005). An implementation of the techniques such as DNA methylation assays to assess epigenetic phenomena, pyrosequencing, RT-PCR are key to further understand the mechanisms involved in the programming of the brain under ED and prenatal Dex exposure.

Early weaning and maternal deprivation in rats have been shown to reduce BDNF levels and suppress adult neurogenesis in the hippocampus (Kikusui et al , 2009; Liu et al., 2000; Mirescu et al., 2004). Therefore, further analysis of the mRNA expression, stress related proteins, oxytocin and serotonin gene transcription and BDNF expression could be evaluated using in situ hybridisation technique.

Over the recent years the concept of resilience has gained attention in stress related research studies (Russo et al., 2012). Neuropeptide Y, a peptide neurotransmitter, is considered to be protective against stress (Morgan et al., 2000). Higher levels of NPY have shown to reduce psychological distress in response to acute stress (Morgan et al., 2002). Therefore, it would be interesting to analyse NPY expression in ED rats as it may explain the differences seen between Wistar and Lewis rats.

7.6 Conclusions

The work presented in the present thesis provides a strong support for the general hypothesis that genetic and environmental factors contribute to the regional brain remodelling and provides further evidence that early life deprivation in rats represents a valuable and interesting model for studying interactions between genetic and environmental effects.

The observed impoverishment of the astroglial morphology in the postnatal deprivation and prenatal dexamethasone treatment can be treated as a demonstration of 'glial plasticity', a phenomenon that deserves more research in the context of stress related pathologies. It is tempting to speculate that enhancing neuronal plasticity and cellular resilience may represent a novel therapeutic approach to the treatment of psychiatric conditions.

Further understanding of the mechanism of action of prenatal corticosteroids in the hippocampus may lead to the discovery of specific treatments preventing undesirable side effects in childrens in whom prenatal corticosteroids were used as a life saving treatment.

Overall, both early postnatal or prenatal manipulations that increase levels of stress and/or glucocorticoids as the chemical mediators of stress, lead to a long-term maladaptive brain remodelling with losses in the hippocampal volume, impoverishment of hippocampal astroglial morphology and changes in the properties of central regulatory receptors in the brain areas involved in the reaction to stress.

These effects are consistent with maladaptive brain plasticity that also implicates astroglia; they are of relevance to stress-related brain disorders.

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Appendix

This section consists of the following information referring to the thesis:

A. **Reagents**
B. **Calculations**
C. **Instruments**
D. **Softwares**
E. **SPSS Statistical Output**
F. **Ethics approval**

A. Reagents

- **Anti-glial fibrillary acidic protein** (anti-GFAP), Monoclonal, clone G-A-5, mouse ascitic fluid, Company: Sigma, Cat. no: G-3893, Lot no: 037K4759, stored at 2-8^{0}C.
- **Anti - S100**, developed in rabbit, IgG fraction of antiserum, Company: Sigma, Product no: S-2644, Lot no: 047K4751, stored at 2-8^{0}C.
- **Anti - PGP 9.5**, Mouse monoclonal neuronal marker, Company: Abcam, Cat no: ab8189, Lot no: 665543, stored at 2-8^{0}C.
- **Anti - Beta III tubulin**, clone 2G10, mouse monoclonal neuronal specific, Company: Abcam, Cat no: ab78078, Lot no: 948254, stored at 2-8^{0}C.
- **Anti Rabbit – FITC**, developed in goat, Company: Sigma-Aldrich, Cat no: F0382, Lot no: 018K60571, stored at 2-8^{0}C.
- **Anti Mouse – Texas red**, developed in goat, Company: Sigma-Aldrich, Cat no: T5393, Lot no: 107K6051, stored at 2-8^{0}C.
- **Albumin from bovine serum**, lyophilized powder, Company: Sigma-Aldrich, Cat no: A4503, mol wt ~66 kDa, stored at 2-8°C
- **Bacitracin**, from bacillus licheniformis, $C_{66}H_{103}N_{17}O_{16}S$, molecular weight: 1422.69, Company: Sigma-Aldrich, Cat no:11702, stored at 2-8°C
- **B27 supplement**, 10ml, Company: Invitrogen, Cat no: 17504-044, stored at -20^{0}C
- **Bovine serum albumin**, 7.5%, Company: Invitrogen UK, Cat. no: 15260, stored at 2-8^{0}C
- **Choline chloride**, (2-Hydroxyethyl) trimethyl ammonium chloride, $(CH_3)_3N(Cl)CH_2CH_2OH$, molecular weight: 139.62, Company: Sigma- Aldrich, Cat no: C7017, stored at cool place.
- **Cover slips**, Company: Fisher brand, 22mm x 50mm, Product no: FB58661.
- **Conical falcon** centrifuge tubes, 15ml, BD Facon^{TM}, BD Biosciences
- **Cell strainer** (Basket), Company: BD Biosciences, UK, Cat no: 352340

- **CytoTox-96** LDH assay, Company: Promega, Southampton, UK, Cat no: G1780, Storage - Substrate Mix and Assay Buffer at –20°C and store LDH Positive Control, Lysis Solution (10X) and Stop Solution at 4°C.

- **Dulbecco's Modified Eagel Medium**: nutrient mixture F12, D-MEM/F12, liquid, 500ml, Company: Invitrogen, Cat. no.: 11320-074, stored at 2-8^{0}C

- **3,3^l – Diaminobenzidine** 99%, 5gms, Company: Sigma-Aldrich, Cat. no: D1,238-4 ; Lot no: 12913PE-179. Stored at 2-8^{0}C

- **Dexamethasone**, powder, 100mg, molecular weight: 392.46, Company: Sigma – Aldrich UK, Cat no: D4902, stored at 2-8^{0}C

- **DAPI** (Vecatshield®), 10 ml, Company: Vector Lab, Cat no. H-1200, concentration of DAPI 1.5 µg/ml, Stored at 2-8^{0}C in the dark.

- **DMSO**, Dimethyl sulfoxide, Hybri-Max™, sterile-filtered, (CH3)2SO, molecular weight: 78.13, Company: Sigma-Aldrich, UK, Cat no: D2650, stored at RT.

- **Ethanol 96%** v/v, C_3H_5OH = 46.07, 2.5L, Company: BDH GPR™, Product no: 28719, Lot no: L142905.

- **EGF** Murine, Company: PeproTech, USA, Cat no: 315-09, stored at -20^{0}C

- **FGF-2** Recombinant Human, Company: PeproTech, Cat no: 100-18B, stored at -20^{0}C

- **Film developer**, D-19 Developer, Kodak Processing chemicals, Cat no: 146 4593, Company: Sigma- Aldrich, Inc. Stored in dark.

- **Film fixative**, Kodak Fixer, Kodak Processing chemicals, Cat no: 197 1746, Company: Sigma-Aldrich, Inc. Stored in dark.

- **Hydrogen peroxide**, 30% w/w solution, FW: 34.01, 500ml, Company: Sigma, Cat. no: H-1009, Lot no: 082K3250, stored at 2-8^{0}C.

- **Histomount™,** Histological mounting medium, 100ml, Company: National diagnostics, Cat. no: HS-103.

- **Hematoxylin solution**, Gill no: 3, 500 ml, Company: Sigma-Aldrich, Cat. no: GHS-3, Lot no: 066K4348.

- **Hyperfilm**, Company: Kodak BioMax MR film, 18x24 cm, Cat no: 873 6936, Carestream Health, Inc. Stored in dark.

- **ImmEdge™ pen**, Company: Vector laboratories, Cat. no: H-4000.

- **L-glutamine** (Gibco) liquid, Company: Invitrogen, Cat. No: 25030-034, stored at 2-8^{0}C

- **Lebovitz's L-15 medium**, Company: Invitrogen, Cat no: 11415-049

- **Magnesium chloride**, anhydrous, molecular weight: 95.21, Company: Sigma-Aldrich, Cat no: M8266, stored at cool place.

- **Methanol**, CH_3OH= 32.04 g/mol, 2.5L, Company: BDH, Product no: 291926G, Lot no: K34702469520.

- **Normal Horse Serum**, 20ml, Company: Vector laboratories, Cat. no: S-2000, stored at 2-8^{0}C.

- **Oxytocin**, lyophilized powder, ~50 IU/mg solid, $C_{43}H_{66}N_{12}O_{12}S_2$, molecular weight: 1007.19, Company: Sigma-Aldrich, Cat no: O3251, stored at 2-8°C.

- **Oxytocin radioligand** - $d(CH_2)_5[Tyr(Me)_2, Thr_4, Orn_8, [^{125}I]\ Tyr_9\text{-}NH_2]$ – [^{125}I]-OVTA, specific activity: 2200 Ci/mmol, concentration: 50 µCi/ml, molecular weight ≈ 1277, Company: American Radiolabeled Chemicals, Inc., Cat no: ARI 0191. Stored at -20^{0}C

- **Oxytocin standards**: [125I]-OVTA microscale standards for quantitative autoradiography Cat no: ARI 0133A, Company: American Radiolabeled Chemicals. Stored at 2-8^{0}C.

- **Optimum cutting tissue** (OCT) embedding medium, Company: RALamb Ltd, Eastbourne, UK

- **Pargyline hydrochloride**, $C_6H_5CH_2N(CH_3)CH_2C{\equiv}CH{\cdot}HCl$, Company: Sigma-Aldrich, UK,Cat no: P8013, molecular weight: 195.69, stored at −20°C.

- **Phosphate buffered saline tablets**, 100 tablets, Company: Oxoid, Product no: BR0014G.

- **Polysine glass slides**, Company: Menzel-Glaser^R, 25x75x1mm.

- **PBS** (Ca^{2+}/Mg^{2+} free), 100ml, Company: Invitrogen, Cat no: 14200-059, stored at RT

- **Poly-D-lysine hydrobromide**, mol wt: 30,000-70,000**,** D-Lys-(D-Lys)n-D-Lys · xHBr Company: Sigma Aldrich, UK Cat no: P7886, stored at -20^{0}C

- **Serotonin radioligand** - [^{3}H]WAY100635, tritium radioligand, Company: Amersham Biosciences, UK, Specific activity: 80 Ci/mmol, radioactive concentration: 200µCi/ml. stored at -20^{0}C

- **Serotonin hydrochloride**, powder form, $C_{10}H_{12}N_2O \cdot HCl$, molecular weight 212.68, Company: Sigma-Alrich UK, Cat no: H9523, stored at 2-8°C.

- **Sodium azide**, 25gms, Company: Sigma-Aldrich, Cat. no: S-2002, Lot no: 098K0052.

- **Triton X-100**, 100ml, Company: Sigma-Aldrich, Cat. no: X-100, Lot no: 058K0073.

- **Tris(hydroxymethyl)aminomethane**, $H_2NC(CH_2OH)_3$, molecular weight: 121.4, Company: Sigma-Aldrich, Cat no: 154563, stored in cool place, hygroscopic in nature.

- **T75** Nunclon flasks, Nuc Roskilde, Denmark, Cat no: 156499.

- **0.05% Trypsin/EDTA**, 500ml, Company: Invitrogen, Cat no: 25300-062, stored at -5 to -20° C.

- **Vectastain Elite ABC kit**, Company: Vector laboratories, Cat. no: PK-6102, contains: Anti-mouse IgG biotinylated Ab (blue label), Normal Horse Serum (Yellow label), Avidin DH-reagent A (grey label), Biotinylated horse-radish peroxidase H- reagent B (grey label).

B. Calculations

- **Phosphate buffered saline (PBS) (pH: 7.4 at RT)**
  For 1 litre of PBS: Dissolve 10 PBS tablets in 1000 ml of distilled water.

- **0.1% PBST - rehydrating solution**
  Mix 5ml of triton X-100 with 500ml of PBS solution in a flask and place on the stirrer to obtain 0.1% PBST.

- **0.5% sodium azide**
  Weigh 0.1 gm of sodium azide and mix with 20ml of PBS to obtain 0.5% sodium azide.

- **1.5% H_2O_2 - peroxidase inactivating solution**
  For 50ml of this solution, mix 10ml of methanol; 0.15ml of 0.3% Triton X-100; 2.5ml of 30% H_2O_2 and then PBS solution was to make up to 50ml.

- **Blocking serum solution (BS)**
  To prepare 10ml of BS with 10% Normal Horse Serum (NHS), mix the following on a vortex: 1ml of NHS; 8ml of 0.1% PBST; 1ml of 0.5% sodium azide solution.

- **Primary antibody (1^0Ab) solution**
  The respective antibody concentration as mentioned in the methodology chapter was diluted using antibody diluent.

- **Primary antibody diluent**
  For 10ml of diluent – 0.1ml of Normal Horse Serum (NHS) + 8.85ml PBS + 1ml of 0.5% sodium azide + 0.05ml of triton X-100 solutions were mixed on vortex.

- **Biotinylated secondary antibody (2^0Ab) solution**
  This is a mixture of horse serum and anti-mouse IgG biotinylated Ab. Solution was made from 4 drops of NHS + 10 ml PBS +1 drop of biotinylated 2^0 Ab stock.

- **Fluorochrome labelled secondary antibody**
  The respective concentration of the Anti rabbit-FITC and Anti mouse-Texas red secondary antibodies labelled with fluorochrome were diluted using PBS.

- **Avidin-Biotin (ABC) complex**
  The Avidin-Biotin complex from Vector laboratories comes as a kit that needs reconstitution. It has: Anti-mouse IgG biotinylated Ab (blue label), Horse normal serum (Yellow label), Avidin DH- reagent A, Biotinylated horse-radish peroxidase H- reagent B.

Mix 10 ml of PBS + 4 drops of reagent A + 4 drops of reagent B. Allow it to stand for 30 minutes.

- **Diaminobenzidine tetra-hydrochloride (DAB)**
  For 10 ml of DAB solution: 9ml of PBS + 1 ml of DAB (already aliquoted).

  DAB aliquots: Each 1 ml aliquots were previously prepared as stock solution containing 1mg DAB/ml of PBS. The aliquots were stored at -25^{0}C.

- **Hematoxylin**
  Ready to use hematoxylin solution was used to stain cell nuclei.

- **Acid - Alcohol differentiation solution**
  1% HCl – 70% ethanol solution was used.
  To make 50ml = 1.18ml of conc HCl + 35ml of 70% ethanol + 13.8ml distilled water.

- **Tris – HCl (50mM, pH 7.4)**
  Weighted 7.55gm of Tris and dissolved in 100ml distilled water. Adjusted pH to 8 and left overnight to settle down. Finally, adjusted pH to 7.4 and diluted to make final volume as 1.25L.

- **Bovine serum albumin (0.1% BSA)**
  In order to prepare 1L of 0.1% BSA = 1gm of BSA was added to 1L of distilled water.

- **Magnesium chloride (10mM)**
  In order to prepare 1L = 0.952gm was added to 1L of distilled water.

- **Bacitracin (0.05%)**
  In order to prepare 1L of 0.05% bacitracin = 0.5gm was added to 1L of distilled water.

- **Choline chloride (100mM)**
  In order to make 1L solution = 13.96gm of choline chloride was dissolved in 1L of distilled water.

- **[^{125}I] OVTA radioligand**

Conversion of fmol

Specific activity – 2200 Ci/mmol

2200 Ci/mmol – 1 mmol → 2200 mCi – 1 µmol → 220µCi – 1 nmol →

2200 nCi – 1 pmol

1 nCi → x? pmol

$x = 1/2200 = 4.54 \times 10^{-4} = 0.000454$ pmol

1 pmol – 1000 fmol

4.54×10^{-4} pmol – y? y = 0.454 fmol

Therefore, 1 nCi → 0.454 fmol

w? nCi → z? **z = 0.454 fmol . w**

- **[^{3}H]WAY100635 radioligand**

80 Ci/mmol = 80 mCi/µmol = 80 µCi/nmol

80 µCi – 1 nmol

200 µCi – x?

x = 2.5 nmol/ml = 2500 nmol/L → 2.5 µM [^{3}H]WAY solution.

Therefore to make 30 ml of 2 nM solution,

2 nM = 2 nmol → 1L

y? → 30ml

y= 0.06 nmol in 30 ml or 60 pmol

This solution: 2.5 nmol/ml i.e. 2.5 pmol/µl

60 pmol → z?

z = 24 µl of [^{3}H]WAY.

Conversion to fmol

Specific activity – 80 Ci/mmol

80 Ci/mmol – 1 mmol → 80 mCi – 1 µmol → 8µCi – 1 nmol →

80 nCi – 1 pmol

1 nCi → x? pmol

x = 1/80 = 0.0125 pmol

1 pmol – 1000 fmol

0.0125 pmol – y? y = 12.5 fmol

Therefore, 1 nCi → 12.5 fmol

w? nCi → z? **z = 12.5 fmol . w**

C. Instruments

- **Brightfield Microscope,** BX51, Olympus with Nicon Coolpix E 4500 video camera, Germany
- **Microscope**, Olympus Inverted Model IMT-2
- **Centrifuge**, Sigma
- **Cryostat**, Model 5040, Bright Instruments Co. Ltd, Huntington, UK
- **Cell counter**, Tamaco.
- **Dell Desktop** Workstation PWS370 computer, Intel Pentium 4, CPU 2.8 GHz
- **Digital colour video camera**, CoolSNAP^{TM}, Photometrics
- **DL10 safelight**, cat no: 1552924, Ilford imaging Ltd., UK
- **Fluorescent microscope**, BX51, Olympus with Nicon Coolpix E 4500 video camera, Germany
- **Heracell 150i CO2 incubator**, Thermo Scientific, UK
- **Herasafe KS12 Safety cabinet,** ThermoScientific, UK
- **Hypercassette^{TM}**, Size 18 x 24 cm, Amersham Biosciences, UK
- **Lightbox**, Northern light imaging research, Inc. Canada
- **Magnetic stirrer thermostat hotplate,** Gallen Kamp, Harding scientific instruments, UK
- **pH meter** 140, Scientific &Medical products Ltd, Corning, UK
- **Vortex**, FISONS, Whirlimixer^{TM}, Serial no: NEL11490, UK
- **XCT scanner**, Stractech Stratec Research SA+ scanner, Stratec Medizintechnik, Pforzheim, Germany

D. Softwares

- **Avizo 5**, version 5, Mercury Computer Systems, Chelmsford, MA, USA.
- **Microsoft Office** packages, Word and Excel 2007, Microsoft corporation, USA
- **MCID**, Version 7.0, Interfocus imaging research, Linton, England, UK.
- **Neurolucida**, Version 9, MicroBrightField, Inc., Germany.
- **SteroInvestigator**, Version 9, MicroBrightField, Inc., Germany.
- **SPSS**, Version 17.0, Statistical software by SPSS Inc., Illinois, USA.
- **View Finder**, 3.0.1 imaging software, Pixera corporation, USA.

E. SPSS Statistical Output

Chapter 3

Brain Weight
Wistar

Multiple Comparisons

Brain Weight
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NH	EH	.10400	.06836	.309	-.0736	.2816
	ED	.11150	.06836	.264	-.0661	.2891
EH	NH	-.10400	.06836	.309	-.2816	.0736
	ED	.00750	.06836	.993	-.1701	.1851
ED	NH	-.11150	.06836	.264	-.2891	.0661
	EH	-.00750	.06836	.993	-.1851	.1701

Lewis

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Brain Weight	Equal variances assumed	.	.	-.270	4	.801	-.02360	.08745	-.26639	.21919
	Equal variances not assumed			.	.	.	-.02360	.	.	.

Brain Volume
Wistar

Multiple Comparisons

Volume
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NH	EH	7.97833	51.50196	.987	-125.7965	141.7532
	ED	77.75500	51.50196	.314	-56.0198	211.5298
EH	NH	-7.97833	51.50196	.987	-141.7532	125.7965
	ED	69.77667	51.50196	.388	-63.9982	203.5515
ED	NH	-77.75500	51.50196	.314	-211.5298	56.0198
	EH	-69.77667	51.50196	.388	-203.5515	63.9982

Lewis

NH vs ED

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Volume	Equal variances assumed	.	.	1.271	5	.260	58.56500	46.08921	-59.91109	177.04109
	Equal variances not assumed			.	.	.	58.56500	.	.	.

Between Strain comparison

ED Group:

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Volume	Equal variances assumed	3.859	.078	-1.695	10	.121	-65.61333	38.71976	-151.88634	20.65967
	Equal variances not assumed			-1.695	7.384	.132	-65.61333	38.71976	-156.21403	24.98736

Left vs Right

Wistar Group

NH

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Volume	Equal variances assumed	.045	.836	1.993	10	.074	.85500	.42893	-.10071	1.81071
	Equal variances not assumed			1.993	9.233	.077	.85500	.42893	-.11158	1.82158

Lewis Group

NH

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Volume	Equal variances assumed	.003	.957	.114	4	.914	.09667	.84469	-2.24856	2.44189
	Equal variances not assumed			.114	3.991	.914	.09667	.84469	-2.25060	2.44394

pQCT analysis

Correlations

		Weight	Volume
Weight	Pearson Correlation	1	.871**
	Sig. (2-tailed)		.000
	N	30	30
Volume	Pearson Correlation	.871**	1
	Sig. (2-tailed)	.000	
	N	30	30

**. Correlation is significant at the 0.01 level (2-tailed).

Regression Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.871a	.758	.749	51.82173

a. Predictors: (Constant), Weight

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	235342.643	1	235342.643	87.635	,000a
	Residual	75193.761	28	2685.491		
	Total	310536.405	29			

a. Predictors: (Constant), Weight

b. Dependent Variable: Volume

Group Comparison
Dorsal Hippocampus volume

Multiple Comparisons

Volume
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NH	EH	2.77500*	.73163	.005	.8746	4.6754
	ED	4.64667*	.73163	.000	2.7463	6.5471
EH	NH	-2.77500*	.73163	.005	-4.6754	-.8746
	ED	1.87167	.73163	.054	-.0287	3.7721
ED	NH	-4.64667*	.73163	.000	-6.5471	-2.7463
	EH	-1.87167	.73163	.054	-3.7721	.0287

*. The mean difference is significant at the 0.05 level.

HX cell counts
CA3

Multiple Comparisons

HX Cell Counts
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NH	EH	39379.60000	41534.61006	.619	-68505.3141	147264.5141
	ED	33530.02667	41534.61006	.704	-74354.8875	141414.9408
EH	NH	-39379.60000	41534.61006	.619	-147264.5141	68505.3141
	ED	-5849.57333	41534.61006	.989	-113734.4875	102035.3408
ED	NH	-33530.02667	41534.61006	.704	-141414.9408	74354.8875
	EH	5849.57333	41534.61006	.989	-102035.3408	113734.4875

GFAP +ve Cell count
CA3

Multiple Comparisons

GFAP +ve Cell Counts
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NH	EH	4.68782	3.59120	.414	-4.6402	14.0159
	ED	16.41897*	3.59120	.001	7.0909	25.7470
EH	NH	-4.68782	3.59120	.414	-14.0159	4.6402
	ED	11.73115*	3.59120	.014	2.4031	21.0592
ED	NH	-16.41897*	3.59120	.001	-25.7470	-7.0909
	EH	-11.73115*	3.59120	.014	-21.0592	-2.4031

Primary Process Length
CA1

Multiple Comparisons

Primary Process Length

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NH	EH	775.78333	437.28084	.212	-360.0406	1911.6073
	ED	2290.50000*	437.28084	.000	1154.6760	3426.3240
EH	NH	-775.78333	437.28084	.212	-1911.6073	360.0406
	ED	1514.71667*	437.28084	.009	378.8927	2650.5406
ED	NH	-2290.50000*	437.28084	.000	-3426.3240	-1154.6760
	EH	-1514.71667*	437.28084	.009	-2650.5406	-378.8927

Mean Primary Process Length
DG

Primary Process Mean Length

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NH	EH	2.51667	1.23482	.137	-.6907	5.7241
	ED	4.60000*	1.23482	.005	1.3926	7.8074
EH	NH	-2.51667	1.23482	.137	-5.7241	.6907
	ED	2.08333	1.23482	.242	-1.1241	5.2907
ED	NH	-4.60000*	1.23482	.005	-7.8074	-1.3926
	EH	-2.08333	1.23482	.242	-5.2907	1.1241

Number of Primary Processes
CA2

Number of Primary Processes

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NH	EH	-2.667	11.408	.970	-32.30	26.97
	ED	-.333	11.408	1.000	-29.97	29.30
EH	NH	2.667	11.408	.970	-26.97	32.30
	ED	2.333	11.408	.977	-27.30	31.97
ED	NH	.333	11.408	1.000	-29.30	29.97
	EH	-2.333	11.408	.977	-31.97	27.30

Chapter 4

Brain weight

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
Weight	Control	6	1.74267	.040928	.016709
	DEX	6	1.77117	.077005	.031437

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Weight	Equal variances assumed	4.289	.065	-.801	10	.442	-.028500	.035602	-.107825	.050825
	Equal variances not assumed			-.801	7.616	.448	-.028500	.035602	-.111323	.054323

Brain volume

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Volume	Equal variances assumed	3.535	.089	-.927	10	.376	-31.05333	33.51364	-105.72637	43.61971
	Equal variances not assumed			-.927	8.385	.380	-31.05333	33.51364	-107.72187	45.61520

Dorsal Hippocampus volume

Group Comparison:

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
Volume	Control	6	32.8179	1.12080	.45757
	DEX treated	6	30.7742	1.22446	.49988

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Volume	Equal variances assumed	.011	.918	3.016	10	.013	2.04375	.67768	.53379	3.55371
	Equal variances not assumed			3.016	9.923	.013	2.04375	.67768	.53219	3.55531

Relative values as % total brain volume:

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
Dorsal Hippocampus Volume (as % Total Brain Volume)	Control	6	1.99031	.096330	.039326
	DEX	6	1.83317	.106496	.043477

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Dorsal Hippocampus Volume (as % Total Brain Volume)	Equal variances assumed	.332	.577	2.680	10	.023	.157140	.058624	.026517	.287763
	Equal variances not assumed			2.680	9.901	.023	.157140	.058624	.026339	.287940

Basolateral Amygdala volume

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
Volume	Control	6	.8094	.05807	.02371
	DEX Treated	6	.8032	.03982	.01626

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Volume	Equal variances assumed	.833	.383	.215	10	.834	.00618	.02874	-.05787	.07022
	Equal variances not assumed			.215	8.851	.835	.00618	.02874	-.05902	.07137

Nucleus Accumbens Volume

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
Volume	Control	6	2.2754	.15708	.06413
	DEX Treated	6	2.2633	.18355	.07493

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Volume	Equal variances assumed	.309	.591	.123	10	.905	.01208	.09863	-.20767	.23184
	Equal variances not assumed			.123	9.767	.905	.01208	.09863	-.20838	.23255

GFAP Stained – Astroglia Cell Counts
CA1

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
GFAP Cell Counts	Equal variances assumed	2.565	.140	.531	10	.607	5807.14000	10937.48894	-18563.10406	30177.38406
	Equal variances not assumed			.531	8.648	.609	5807.14000	10937.48894	-19089.37420	30703.65420

Primary Process Length
CA3

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Primary Process Length	Equal variances assumed	.070	.797	6.855	10	.000	4525.08333	660.15825	3054.15908	5996.00758
	Equal variances not assumed			6.855	9.993	.000	4525.08333	660.15825	3054.02095	5996.14572

Primary process mean length
CA2

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Primary Process Mean Length	Equal variances assumed	.621	.449	1.914	10	.085	1.25000	.65324	-.20551	2.70551
	Equal variances not assumed			1.914	8.881	.088	1.25000	.65324	-.23076	2.73076

Number of primary processes

DG

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Number of Primary Processes	Equal variances assumed	10.355	.009	.454	10	.660	6.833	15.051	-26.703	40.370
	Equal variances not assumed			.454	6.075	.666	6.833	15.051	-29.885	43.552

Chapter 5

Cg

Dependent Variable:5HT-1A Receptor Binding

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.002[a]	3	.001	8.514	.001
Intercept	.011	1	.011	156.533	.000
Sex	6.202E-6	1	6.202E-6	.091	.766
Treatment	.002	1	.002	24.383	.000
Sex * Treatment	7.315E-5	1	7.315E-5	1.069	.313
Error	.001	20	6.842E-5		
Total	.014	24			
Corrected Total	.003	23			

5HT-1A Receptor Binding
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Male Control	Male Dex	-.020167*	.004776	.002	-.03353	-.00680
	Female Control	-.002475	.004776	.954	-.01584	.01089
	Female Dex	-.015658*	.004776	.018	-.02903	-.00229
Male Dex	Male Control	.020167*	.004776	.002	.00680	.03353
	Female Control	.017692*	.004776	.007	.00432	.03106
	Female Dex	.004508	.004776	.782	-.00886	.01788
Female Control	Male Control	.002475	.004776	.954	-.01089	.01584
	Male Dex	-.017692*	.004776	.007	-.03106	-.00432
	Female Dex	-.013183	.004776	.054	-.02655	.00018
Female Dex	Male Control	.015658*	.004776	.018	.00229	.02903
	Male Dex	-.004508	.004776	.782	-.01788	.00886
	Female Control	.013183	.004776	.054	-.00018	.02655

BLA

Dependent Variable:5HT-1A Receptor Binding

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.001[a]	3	.000	3.711	.028
Intercept	.007	1	.007	104.906	.000
Sex	1.380E-5	1	1.380E-5	.218	.646
Treatment	.000	1	.000	7.092	.015
Sex * Treatment	.000	1	.000	3.825	.065
Error	.001	20	6.342E-5		
Total	.009	24			
Corrected Total	.002	23			

5HT-1A Receptor Binding
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Male Control	Male Dex	-.002300	.004598	.958	-.01517	.01057
	Female Control	.007875	.004598	.343	-.00499	.02074
	Female Dex	-.007142	.004598	.426	-.02001	.00573
Male Dex	Male Control	.002300	.004598	.958	-.01057	.01517
	Female Control	.010175	.004598	.154	-.00269	.02304
	Female Dex	-.004842	.004598	.721	-.01771	.00803
Female Control	Male Control	-.007875	.004598	.343	-.02074	.00499
	Male Dex	-.010175	.004598	.154	-.02304	.00269
	Female Dex	-.015017*	.004598	.019	-.02789	-.00215
Female Dex	Male Control	.007142	.004598	.426	-.00573	.02001
	Male Dex	.004842	.004598	.721	-.00803	.01771
	Female Control	.015017*	.004598	.019	.00215	.02789

D Hippo CA3

Dependent Variable:5HT-1A Receptor Binding

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.001[a]	3	.000	2.204	.119
Intercept	.116	1	.116	794.046	.000
Sex	3.338E-6	1	3.338E-6	.023	.881
Treatment	.001	1	.001	6.488	.019
Sex * Treatment	1.481E-5	1	1.481E-5	.102	.753
Error	.003	20	.000		
Total	.119	24			
Corrected Total	.004	23			

5HT-1A Receptor Binding
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Male Control	Male Dex	-.014117	.006965	.212	-.03361	.00538
	Female Control	-.000825	.006965	.999	-.02032	.01867
	Female Dex	-.011800	.006965	.353	-.03130	.00770
Male Dex	Male Control	.014117	.006965	.212	-.00538	.03361
	Female Control	.013292	.006965	.256	-.00620	.03279
	Female Dex	.002317	.006965	.987	-.01718	.02181
Female Control	Male Control	.000825	.006965	.999	-.01867	.02032
	Male Dex	-.013292	.006965	.256	-.03279	.00620
	Female Dex	-.010975	.006965	.414	-.03047	.00852
Female Dex	Male Control	.011800	.006965	.353	-.00770	.03130
	Male Dex	-.002317	.006965	.987	-.02181	.01718
	Female Control	.010975	.006965	.414	-.00852	.03047

V Hippo DG

Dependent Variable:Oxytocin Receptor Binding

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.234[a]	3	.411	1.608	.219
Intercept	154.388	1	154.388	603.615	.000
Sex	.256	1	.256	.999	.329
Treatment	.015	1	.015	.060	.810
Sex * Treatment	.963	1	.963	3.765	.067
Error	5.115	20	.256		
Total	160.737	24			
Corrected Total	6.349	23			

Oxytocin Receptor Binding
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Male Control	Male Dex	.35019	.29199	.634	-.4671	1.1674
	Female Control	.60702	.29199	.194	-.2102	1.4243
	Female Dex	.15597	.29199	.950	-.6613	.9732
Male Dex	Male Control	-.35019	.29199	.634	-1.1674	.4671
	Female Control	.25684	.29199	.815	-.5604	1.0741
	Female Dex	-.19421	.29199	.909	-1.0115	.6230
Female Control	Male Control	-.60702	.29199	.194	-1.4243	.2102
	Male Dex	-.25684	.29199	.815	-1.0741	.5604
	Female Dex	-.45105	.29199	.431	-1.2683	.3662
Female Dex	Male Control	-.15597	.29199	.950	-.9732	.6613
	Male Dex	.19421	.29199	.909	-.6230	1.0115
	Female Control	.45105	.29199	.431	-.3662	1.2683

STr

Dependent Variable:Oxytocin Receptor Binding

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	80.839[a]	3	26.946	6.733	.003
Intercept	13489.825	1	13489.825	3370.777	.000
Sex	78.784	1	78.784	19.686	.000
Treatment	.737	1	.737	.184	.672
Sex * Treatment	1.318	1	1.318	.329	.572
Error	80.040	20	4.002		
Total	13650.704	24			
Corrected Total	160.879	23			

Oxytocin Receptor Binding
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Male Control	Male Dex	-.81916	1.15499	.892	-4.0519	2.4136
	Female Control	3.15490	1.15499	.057	-.0778	6.3876
	Female Dex	3.27321*	1.15499	.047	.0405	6.5059
Male Dex	Male Control	.81916	1.15499	.892	-2.4136	4.0519
	Female Control	3.97406*	1.15499	.013	.7413	7.2068
	Female Dex	4.09237*	1.15499	.010	.8596	7.3251
Female Control	Male Control	-3.15490	1.15499	.057	-6.3876	.0778
	Male Dex	-3.97406*	1.15499	.013	-7.2068	-.7413
	Female Dex	.11830	1.15499	1.000	-3.1144	3.3510
Female Dex	Male Control	-3.27321*	1.15499	.047	-6.5059	-.0405
	Male Dex	-4.09237*	1.15499	.010	-7.3251	-.8596
	Female Control	-.11830	1.15499	1.000	-3.3510	3.1144

VMH

Dependent Variable:Oxytocin Receptor Binding

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	153.392[a]	3	51.131	20.400	.000
Intercept	2474.848	1	2474.848	987.388	.000
Sex	118.505	1	118.505	47.280	.000
Treatment	17.756	1	17.756	7.084	.015
Sex * Treatment	17.131	1	17.131	6.835	.017
Error	50.129	20	2.506		
Total	2678.370	24			
Corrected Total	203.521	23			

Oxytocin Receptor Binding
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Male Control	Male Dex	.03057	.91405	1.000	-2.5278	2.5889
	Female Control	-6.13391*	.91405	.000	-8.6923	-3.5755
	Female Dex	-2.72391*	.91405	.034	-5.2823	-.1655
Male Dex	Male Control	-.03057	.91405	1.000	-2.5889	2.5278
	Female Control	-6.16448*	.91405	.000	-8.7228	-3.6061
	Female Dex	-2.75448*	.91405	.032	-5.3128	-.1961
Female Control	Male Control	6.13391*	.91405	.000	3.5755	8.6923
	Male Dex	6.16448*	.91405	.000	3.6061	8.7228
	Female Dex	3.40999*	.91405	.007	.8516	5.9684
Female Dex	Male Control	2.72391*	.91405	.034	.1655	5.2823
	Male Dex	2.75448*	.91405	.032	.1961	5.3128
	Female Control	-3.40999*	.91405	.007	-5.9684	-.8516

Chapter 6

Proliferation

Dependent Variable:No of cells

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	771.719[a]	7	110.246	7.448	.000
Intercept	26854.031	1	26854.031	1814.206	.000
Groups	306.281	1	306.281	20.692	.000
Treatment	459.344	3	153.115	10.344	.000
Groups * Treatment	6.094	3	2.031	.137	.937
Error	355.250	24	14.802		
Total	27981.000	32			
Corrected Total	1126.969	31			

No of cells - Proliferation
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	0	4.000	2.196	.311	-2.52	10.52
	1	5.500	2.196	.110	-1.02	12.02
	10	10.250*	2.196	.003	3.73	16.77
0	Control	-4.000	2.196	.311	-10.52	2.52
	1	1.500	2.196	.902	-5.02	8.02
	10	6.250	2.196	.062	-.27	12.77
1	Control	-5.500	2.196	.110	-12.02	1.02
	0	-1.500	2.196	.902	-8.02	5.02
	10	4.750	2.196	.189	-1.77	11.27
10	Control	-10.250*	2.196	.003	-16.77	-3.73
	0	-6.250	2.196	.062	-12.77	.27
	1	-4.750	2.196	.189	-11.27	1.77

Differentiation – GFAP cells

Dependent Variable:GFAP cells

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	56.875[a]	7	8.125	2.671	.034
Intercept	1596.125	1	1596.125	524.753	.000
Groups	.125	1	.125	.041	.841
Treatment	8.125	3	2.708	.890	.460
Groups * Treatment	48.625	3	16.208	5.329	.006
Error	73.000	24	3.042		
Total	1726.000	32			
Corrected Total	129.875	31			

GFAP cells
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	0	3.000	1.109	.078	-.29	6.29
	1	3.250	1.109	.053	-.04	6.54
	10	4.250*	1.109	.011	.96	7.54
0	Control	-3.000	1.109	.078	-6.29	.29
	1	.250	1.109	.996	-3.04	3.54
	10	1.250	1.109	.680	-2.04	4.54
1	Control	-3.250	1.109	.053	-6.54	.04
	0	-.250	1.109	.996	-3.54	3.04
	10	1.000	1.109	.804	-2.29	4.29
10	Control	-4.250*	1.109	.011	-7.54	-.96
	0	-1.250	1.109	.680	-4.54	2.04
	1	-1.000	1.109	.804	-4.29	2.29

LDH Assay

Dependent Variable:LDH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1109.602[a]	7	158.515	2.848	.039
Intercept	188495.670	1	188495.670	3386.493	.000
Group	30.252	1	30.252	.544	.472
Treatment	1054.955	3	351.652	6.318	.005
Group * Treatment	24.395	3	8.132	.146	.931
Error	890.577	16	55.661		
Total	190495.849	24			
Corrected Total	2000.179	23			

LDH
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	0	.098667	.035421	.090	-.01476	.21210
	1	.095333	.035421	.103	-.01810	.20876
	10	.090667	.035421	.124	-.02276	.20410
0	Control	-.098667	.035421	.090	-.21210	.01476
	1	-.003333	.035421	1.000	-.11676	.11010
	10	-.008000	.035421	.996	-.12143	.10543
1	Control	-.095333	.035421	.103	-.20876	.01810
	0	.003333	.035421	1.000	-.11010	.11676
	10	-.004667	.035421	.999	-.11810	.10876
10	Control	-.090667	.035421	.124	-.20410	.02276
	0	.008000	.035421	.996	-.10543	.12143
	1	.004667	.035421	.999	-.10876	.11810